FEE TRANSMITTAL for FY 2005

(Large Entity)

	Complete if Known
Application Number	09/308,223
Filing Date	August 12, 1999
First Named Inventor	Georg KALLMEYER et al
Examiner Name	Brandon J. Fetterolf
Group Art Unit	1642
☐ Applicant claims small entity status	Attorney Docket Number: 2924-139
Total Amount of Payment ($) **$500**	Confirmation Number: 5876

METHOD OF PAYMENT (check one)

1. ☒ The Commissioner is hereby authorized to charge the fees indicated below or credit overpayment to Deposit Account Number 02-2135 in the name of Rothwell, Figg, Ernst & Manbeck

   ☒ Charge any additional fee required under 37 CFR 1.16 and 1.17 to Deposit Account No. 02-2135.

2. ☐ Payment by check enclosed

FEE CALCULATION

1. FILING, SEARCH AND EXAMINATION FEES

Code	Fee	Fee Description	Fee Paid
1001	300	Utility Filing Fee	[ ]
	790	*filed before Dec. 8, 2004*	[ ]
1111	500	Utility Search Fee	[ ]
1311	200	Utility Examination Fee	[ ]
1002	200	Design Filing Fee	[ ]
	350	*filed before Dec. 8, 2004*	[ ]
1112	100	Design Search Fee	[ ]
1312	130	Design Examination Fee	[ ]
1003	200	Plant Filing Fee	[ ]
	550	*filed before Dec. 8, 2004*	[ ]
1113	300	Plant Search Fee	[ ]
1313	160	Plant Examination Fee	[ ]
1004	300	Reissue Filing Fee	[ ]
	790	*filed before Dec. 8, 2004*	[ ]
1114	500	Reissue Search Filing Fee	[ ]
1314	600	Reissue Examination Fee	[ ]
1005	200	Provisional Filing Fee	[ ]

SUBTOTAL $

2. CLAIMS

			Extra Claims		Fee	Fee Paid
Total Claims	[ ]	- 20* =	[ ]	x	$50 =	[ ]
Independent Claims	[ ]	- 3* =	[ ]	x	200 =	[ ]
Multiple Dependent Claims				+	360 =	[ ]

**or number previously paid, if greater*

SUBTOTAL $

3. APPLICATION SIZE FEE

Total Sheets [ ] - 100 = [ ]/50 = [ ]** x $250 =

*** Number of each additional 50 or fraction thereof*

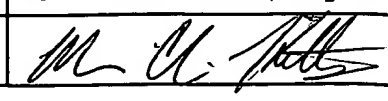
SUBTOTAL $

FEE CALCULATION (continued)

4. ADDITIONAL FEES

Fee Code	Fee Paid	Fee Description	Fee Paid
1051	130	Surcharge - late filing fee or oath	[ ]
1052	50	Surcharge - late provisional filing fee or cover sheet	[ ]
1053	130	Non-English specification	[ ]
1812	2,520	For filing a request for reexamination	[ ]
1804	920	Requesting publication of SIR prior to Examiner action	[ ]
1805	1,840	Requesting publication of SIR after Examiner action (reduced by basic filing fee paid)	[ ]
1251	120	Extension for reply within first month	[ ]
1252	450	Extension for reply within second month	[ ]
1253	1,020	Extension for reply within third month	[ ]
1254	1,590	Extension for reply within fourth month	[ ]
1255	2,160	Extension for reply within fifth month	[ ]
1401	500	Notice of Appeal	[ ]
1402	500	Filing a brief in support of an appeal	[$500]
1403	1,000	Request for Oral Hearing	[ ]
1451	1,510	Petition to institute a public use proceeding	[ ]
1452	500	Petition to revive -unavoidable	[ ]
1453	1,500	Petition to revive - unintentional	[ ]
1807	50	Processing fee under 37 CFR 1.17(q)	[ ]
1806	180	Submission of Information Disclosure Statement	[ ]
1809	790	Filing a submission after final rejection (37 CFR .129(a))	[ ]
1810	790	For each additional invention to be examined (37 CFR 1.129(b))	[ ]
1801	790	Request for Continued Examination (RCE)	[ ]
1802	900	Request for expedited examination of a design application	[ ]
1504	300	Publication fee for early, voluntary, or normal publication	[ ]
1505	300	Publication fee for republication	[ ]
1455	200	Filing application for patent term adjustment	[ ]
1456	400	Request for reinstatement of term reduced	[ ]
1814	130	Statutory Disclaimer	[ ]
Other fee (specify)			[ ]

SUBTOTAL $ 500

SUBMITTED BY				Complete (if applicable)	
NAME AND REG. NUMBER	Monica Chin Kitts, Reg. No. 36,105				
SIGNATURE		DATE	July 28, 2006	DEPOSIT ACCOUNT USER ID	

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE TO THE BOARD OF PATENT APPEALS AND INTERFERENCES

Appl. No.	: 09/308,223
Applicant	: Georg KALLMEYER et al.
Filed	: August 12, 1999
Title:	: STABLE LYOPHILIZED PHARMACEUTICAL SUBSTANCES FROM MONOCLONAL OR POLYCLONAL ANTIBODIES
TC/A.U.	: 1642
Examiner	: Brandon J. Fetterolf
Docket No.	: 2924-139
Customer No.	: 6449
Confirmation No.	: 5876

Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

July 28, 2006

APPELLANT'S BRIEF ON APPEAL UNDER 37 C.F.R. §41.37

Sir:

The following comprises the Applicant's Brief on Appeal from the Office Action dated November 1, 2005, in which claims 13, 15-18 and 22-36, were finally rejected. A Notice of Appeal was filed April 28, 2006 along with a Pre-Appeal Brief Request for Review and a petition for a three-month extension of time. A decision on the Pre-Appeal Brief Request was mailed on May 24, 2006 stating that at least one issue remains for appeal. This Appeal Brief is accompanied by the required Appeal fee set forth in 37 C.F.R. § 41.20(b)(2), along with a petition for an extension of time, and is being timely filed.

I.
REAL PARTY IN INTEREST

The owner of the above-referenced patent application and the real party in interest in this appeal is Roche Diagnostics GmbH, Federal Republic of Germany.

II.
RELATED APPEALS AND INTERFERENCES

The Applicant is unaware of any other appeals or interferences related to the subject matter of this appeal.

III.
STATUS OF CLAIMS

Claims 13, 15-18 and 22-36 are pending in the application. Claims 13, 15-18 and 22-36 were rejected in the Final Office Action dated November 1, 2005. Claims 1-12, 14, and 19-21 have been canceled. Applicant appeals from the rejection of claims 13, 15-18 and 22-36. The appealed claims are reproduced in the Appendix attached hereto.

IV.
STATUS OF AMENDMENTS

No Amendments have been submitted since the final rejection dated November 1, 2005.

V.

SUMMARY OF THE CLAIMED SUBJECT MATTER

The present invention is directed to lyophilized pharmaceutical preparations of monoclonal or polyclonal antibodies. The lyophilized preparations contain an amino sugar, at least one amino acid; and a surfactant, but does not contain polyethylene glycols or additional proteins.

Monoclonal and polyclonal antibodies are increasingly important for therapeutic and diagnostic purposes and methods for the stabilization of lyophilized antibodies are known in the art (Specification page 2, line 18 to page 5, line 26). However, the known methods for stabilization of antibodies often require stabilizers which are not acceptable from a medical point of view (Specification page 5, line 27 to page 6, line 22). Polymers (such as polyethylene glycol and gelatin) and proteins (such as serum albumin) pose a risk due to their origin (e.g. viral contamination) and can cause an allergic reaction (Specification page 5, line 30 to page 6, line 4).

The present inventors have surprisingly found that stable pharmaceutical lyophilisates of monoclonal or polyclonal antibodies are obtained when the preparation contains the antibody, an amino sugar, at least one amino acid, and a surfactant (Specification page 7, lines 3-9). These preparations according to the present invention are physiologically well tolerated, have a relatively simple composition and can be dosed exactly (Specification page 7, lines 12-14). The preparations exhibit no detectable degradation products when subjected to multiple freezing and thawing cycles or during long term storage (Specification page 7, lines 14-17). The claimed

preparations exhibit no particle formation (i.e. turbidity) after reconstitution with water (Specification page 8, lines 10-13).

VI.

GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The only issue on appeal is whether claims 13, 15-18 and 22-36 are unpatentable under 35 U.S.C. § 103(a) as obvious over Andya in view of Michaelis.

VII.

ARGUMENT

Claims 13, 15-18 and 22-36 are not obvious over Andya in view of Michaelis because they recite subject matter not shown or suggested by Andya in view of Michaelis.

Andya in view of Michaelis fails to render obvious any of claims 13, 15-18 and 22-36. Claims 13, 15-18 and 22-36 are directed to a lyophilizate which contains a monoclonal or polyclonal antibody, an amino sugar, at least one amino acid, and a surfactant, where the lyophilizate does not contain polyethylene glycols or additional proteins; a composition containing the lyophilizate and a method for preparing the lyophilizate. The office actions contend that one skilled in the art would be motivated to combine Andya and Michaelis to arrive at the present invention because both of these references teach the preparation of stable pharmaceutical compositions. Applicants

respectfully point out that Michaelis discloses the addition of an amino sugar to stabilize a protein which is a member of the four-helix bundle class of cytokines (G-CSF) not antibodies while Andya is directed to antibody preparations which do not contain amino sugars. Antibodies and cytokines are in different protein classes and have different structures and therefore different stabilization requirements.

The Office Action of November 1, 2005 states that it would have been obvious to modify the lyophilizate of Andya to include an amino sugar as taught by Michaelis. Michaelis discloses the addition of an amino sugar to stabilize G-CSF protein not antibodies. Applicants respectfully point out that it is known that different protein classes required different stabilizers, not all stabilizers are suitable for all proteins. Osterberg (WO 94/07510) states on page 4, lines 25-32 that:

"Proteins are different with regard to physico-chemical properties. When preparing a pharmaceutical preparation which should be physico-chemical acceptable, and stable for a long time, consideration cannot only be taken to the physiological properties of the protein but also other aspects must be considered such as the industrial manufacture, easy handling for the patient and safety for the patient. **The results of these aspects are not predictable when testing different formulations and there often is a unique solution for each protein**," (emphasis added).

Osterberg points out that different proteins are different in their physico-chemical properties and thus for each protein or class of proteins an individual solution has to be developed and thus it cannot be predicted that the same formulation will be useful for a different class of protein.

Manning (Pharmaceutical Research, Vol. 6, No. 11, 1989, p. 903-918) is a general article related to the stability of proteinaceous pharmaceuticals. On page 913, left column, first sentence of the last paragraph, it is stated that "protein stability encompasses many complicated and interrelated chemical and physical processes". From this it can be concluded that for every protein or class of proteins an individual solution has to be found due to different physical and chemical constraints. Thus, one skilled in the art would not extrapolate the disclosure in Michaelis to any and all protein classes and certainly not to any and all pharmaceutical compositions as suggested in the office actions.

Osterberg's and Manning's conclusions are supported by the fact that different substances are indicated as good stabilizers in some references and as not useful as a stabilizer in other references. For example, Kunihiro (EP 0 689 843) page 4, line 4 - 7, indicates that the combination of soluble thrombomodulin together with albumin, purified gelatin, glycine, glucose or mannitol **failed** to exhibit sufficient long term stability. Thus, this document contradicts the contention in the office actions that Michaelis' teaching can be applied to any and all pharmaceutical preparations. Kunihiro teaches away from the current invention in that the combination of an amino acid with a sugar had no beneficial effect on stability.

Hanson (chapter 7 in Stability of Protein Pharmaceuticals, 1992) indicates on page 217, second paragraph, line 6 to 7 that "Ornithine, aspartic acid, glutamic acid, alanine and glycine did not stabilize" intravenous immunoglobulin preparations.

Thus, Hanson also contradicts the contention in the office action that Michaelis' teaching can be applied to any and all pharmaceutical preparations and teaches away from the current invention which shows that the use of the amino acids listed in Hanson improve the stability of the lyophilized antibody formulation.

Metzner (EP 0 733 702) which is equivalent to US Patent No. 6,204,036 indicates that histidine and glutamic acid alone, even without further additives, show sufficient stabilization (page 3, line 9, of the German text, column 5, lines 56-58 of the US text). In contrast to Metzner, Michaelis (WO 94/14465) states on page 10, lines 4 to 7 of the German WO 94/14465 that the addition of glutamic acid has no significant impact on the storage stability. Both Metzner and Michaelis also indicate that the surfactant had no impact on storage stability (Metzner page 3, lines 42-43 or col. 6, lines 48-50 in the U.S. Patent, Michaelis page 9, last paragraph of WO 94/14465) but the present inventors have found that the surfactant does affect stability in the present invention. Thus, prior art formulations for stabilizing different pharmaceutical preparations clearly cannot be generalized.

Nema (J. Parent Sci. Technol., 47, p. 76-83, 1993) states on page 81, left column, last sentence of the first paragraph: "A surprising result was obtained with trehalose, a disaccharide which is considered by many workers to be one of the best cryoprotectants, but proved to be ineffective in this study at a concentration of 5%w/v". This statement also supports the conclusion of the non-transferability of formulations to different classes of proteins.

In view of the above discussed references, three conclusions can be drawn.

1) There is no suggestion that combining different compounds discussed in different references will result in a formulation with further improved stability. Furthermore there is no suggestion in these documents as to the particular combination of compounds as described in the current invention.

2) As can be seen from these references, it is not possible to generally transfer the known composition of a formulation useful with one class of proteins or with one protein to other proteins. It was not probable or predictable that a formulation for stabilizing a cytokine would be successful with the antibody preparation of the present invention.

3) There are no cited documents that suggest or disclose that a formulation for stabilizing a non-antibody protein can be used for the stabilization of a lyophilized antibody preparation.

Applicants also point out that Andya teaches at column 29, lines 49-50, that "reducing sugars are not suitable as lyoprotectants for the antibody". In contrast to this, the present inventors have found that amino sugars derived from reducing sugars such as glucose or galactose are suitable for use in the present invention.

Applicants contend that one skilled in the art would not expect Michaelis' formulation to be useful for any and all pharmaceutical preparations as different protein classes require different stabilization agents and there is no reason to believe that Michaelis' formulation would stabilize antibody preparations such as Andya's antibody formulation.

Conclusion

For all of the above noted reasons, it is strongly contended that certain clear differences exist between the present invention as claimed in claims 13, 15-18 and 22-36 and the prior art relied upon by the Examiner. It is further contended that these differences are more than sufficient evidence that the present invention would not have been obvious to a person having ordinary skill in the art at the time the invention was made.

This final rejection being in error, therefore, it is respectfully requested that this honorable Board of Patent Appeals and Interferences reverse the Examiner's decision in this case and indicate the allowability of application claims 13, 15-18 and 22-36.

In the event that this paper is not being timely filed, the applicant respectfully petitions for an appropriate extension of time. Please charge any fee or credit any overpayment pursuant to 37 §C.F.R. 1.16 or §1.17 to Deposit Account No. 02-2135.

Respectfully submitted,

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VIII.

APPENDIX OF CLAIMS ON APPEAL

Claims 1-12 (Canceled).

13. A lyophilizate, comprising

(a) a monoclonal antibody or a polyclonal antibody;

(b) an amino sugar;

(c) at least one amino acid; and

(d) a surfactant,

wherein the lyophilzate contains no poly ethylene glycols or additional proteins.

14. (Cancelled).

15. The lyophilizate of claim 13, wherein the lyophilizate contains a single amino acid or two different amino acids.

16. The lyophilizate of claim 13, further comprising a buffering agent or an isotonizing agent which is present in an amount such that a reconstituted solution of the lyophilizate has a pH value of 5-8.

17. The lyophilizate of claim 13, wherein the lyophilizate is storage-stable for a time period of at least three months at a temperature of about 4-12°C.

18. The lyophilizate of claim 13, wherein the lyophilizate is storage-stable for a time period of at least three months at a temperature of about 18-23°C.

Claims 19-21 (Canceled).

22. The lyophilizate of claim 13, wherein the amino sugar comprises at least one member selected from the group consisting of glucosamine, N-methyl-glucosamine, galactosamine and neuraminic acid.

23. The lyophilizate of claim 13, wherein the amino acid comprises at least one member selected from the group consisting of arginine, lysine, histidine, ornithine, glutamic acid, aspartic acid, isoleucine, leucine, alanine, phenylalanine, tyrosine and tryptophan.

24. The lyophilizate of claim 13, wherein the surfactant comprises a polysorbate or a polyoxyethylene-polyoxypropylene polymer.

25. The lyophilizate of claim 13, wherein the monoclonal antibody or the polyclonal antibody has a molecular weight of 50-200 kDa per monomer unit.

26. The lyophilizate of claim 13, wherein the monoclonal antibody or the polyclonal antibody is directed against an antigen selected from the group consisting of hepatitis B virus, AIDS virus, cytomegalovirus, meningoencephalitis virus, rubella virus, measles virus, rabies pathogen, Pseudomonas aeruginosa, varicella-zoster virus, tetanus pathogen, van Willebrandt factor, nerve growth factor receptor, platelet derived growth factor receptor, selectin, integrin and diphtheria pathogen.

27. A lyophilizate, consisting essentially of

(a) a monoclonal antibody or a polyclonal antibody;

(b) an amino sugar;

(c) at least one amino sugar;

(d) a surfactant; and

(e) an inorganic acid as a buffering agent,

wherein the lyophilzate contains no poly ethylene glycols or additional proteins.

28. A liquid pharmaceutical composition comprising the lyophilizate of claim 13 dissolved in a physiologically acceptable solution.

29. The liquid pharmaceutical composition of claim 28, wherein the composition has a pH value of 5-8.

30. The liquid pharmaceutical composition of claim 28, wherein the composition contains 1-10 mg/ml of antibody.

31. The liquid pharmaceutical composition of claim 28, wherein the composition contains up to 200 mg/ml of sugar or amino sugar.

32. The liquid pharmaceutical composition of claim 28, wherein the composition contains up to 100 mg/ml of amino acid.

33. The liquid pharmaceutical composition of claim 28, wherein the composition contains 0.05-0.5 mg/ml of surfactant.

34. A liquid pharmaceutical composition comprising the lyophilizate of claim 27 dissolved in a physiologically acceptable solution.

35. The liquid pharmaceutical composition of claim 30, wherein the composition has a pH value of 5-8.

36. A method of preparing a lyophilizate, the method comprising mixing a buffered solution containing a monoclonal antibody or a polyclonal antibody, an amino acid sugar, at least one amino acid and a surfactant, to prepare a mixed solution, wherein the mixed solution was a pH value of 5-8; and

lyophilizing the mixed solution, wherein the lyophilizate contains no polyethylene glycols or additional proteins.

IX.

EVIDENCE APPENDIX

A copy of the background references discussed above and in applicant's January 31, 2006 response are attached. These references were submitted with the information disclosure statement filed on September 2, 2005.

X.

RELATED PROCEEDINGS APPENDIX

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(54) Title: COMPOSITION COMPRISING COAGULATION FACTOR VIII FORMULATION, PROCESS FOR ITS PREPARATION AND USE OF A SURFACTANT AS STABILIZER

(57) Abstract

The present invention relates to novel composition comprising coagulation factor VIII and a non-ionic surfactant such as block copolymers, e.g. polyoxamers or polyoxyethylene (20) sorbitan fatty acid esters e.g. polysorbate 20 or polysorbate 80 as stabilizer. The composition can also comprise sodium chloride, calcium chloride, L-histidine and/or sugars or sugar alcohols. The invention also relates to the use of a non-ionic surfactant as stabilizer for a composition comprising coagulation factor VIII.

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COMPOSITION COMPRISING COAGULATION FACTOR VIII FORMULATION, PROCESS FOR ITS PREPARATION AND USE OF A SURFACTANT AS STABILIZER.

The present invention relates to a novel formulation comprising coagulation factor VIII and a non-ionic surfactant such as block co-polymers, e.g. polyoxamers or polyoxyethylene (20) sorbitan fatty acid esters e.g. polysorbate 20 or polysorbate 80. The composition can also comprise sodium chloride, calcium chloride, L-histidine and/or sugars and/or sugar alcohols.

Haemophilia is an inherited disease which has been known for centuries but it is only within the last three decades that it has been possible to differentiate between the various forms; haemophilia A, haemophilia B and haemophilia C. Haemophilia A is the most frequent form. It affects only males with an incidence of one or two individuals per 10 000 live-born males. The disease is caused by strongly decreased level or absence of biologically active coagulation factor VIII (antihaemophilic factor) which is a protein normally present in plasma. The clinical manifestation of haemophilia A is a strong bleeding tendency and before treatment with factor VIII concentrates was introduced, the mean age of those patients was less than 20 years. Concentrates of factor VIII obtained from plasma have been available for about three decades. This has improved the situation for treatment of haemophilia patients considerably and given them possibility to live a normal life.

Therapeutic factor VIII concentrates have until now been prepared by fractionation of plasma. However, there are now methods available for production of factor VIII in cell culture using recombinant DNA techniques as reported in e.g. J Gitschier et al. Nature 312, 330-37. 1984 and EP 160 457.

Factor VIII concentrates derived from human plasma contain several fragmented fully active factor VIII forms (Andersson et al, Proc. Natl. Acad. Sci. USA, Vol 83,2979-83, May 1986). The smallest active form has a

molecular mass of 170 kDa and consists of two chains of 90 kDa and 80 kDa held together by a metal ion bridge. Reference is here made to EP 197 901. Kabi Pharmacia has developed a recombinant factor VIII product which corresponds to the 170 kDa plasma factor VIII form in therapeutic factor VIII concentrates. The truncated recombinant factor VIII molecule is termed r-VIII SQ and is produced by Chinese Hamster Ovary (CHO) cells in a cell culture process in serum free medium at finite passage.

The specific activity of r-VIII SQ could be more than 12 000 IU/mg protein and preferably more than 14 000 IU/ mg. Activity of about 15 000 IU/mg has been measured. About 10 000 IU VIII:C per mg protein has earlier been known for our r-VIII SQ.

Recombinant factor VIII SQ is indicated for treatment of classical haemophilia. The dosage is similar to the dosage of the plasma factor VIII concentrates. Due to the high concentration now obtainable only small volumes are needed for injection.

The structure and biochemistry of recombinant factor VIII-products in general have been described by Kaufman Tibtech, Vol 9,1991 and Hematology, 63, 155-65, 1991. The structure and biochemistry of r-VIII SQ have been described in WO 91/09122.

The stability of proteins is generally a problem in pharmaceutical industry. It has often been solved by drying of the protein in different drying processes, such as freeze drying. The protein has thereafter been distributed and stored in dried form.
The solution before drying or freeze-drying, the dried material and the reconstituted product should all be stable, so that not too much activity is lost during the drying process, the storage or during handling.

Factor VIII which has been fractionated from plasma is normally sold as lyophilized powder which should be reconstituted with water.

A formulation with a low amount of protein will generally loose activity during purification, sterile manufacturing, in the package and during the administration. This problem is usually solved by the addition of human

albumin which reduces the activity loss of the active protein considerably. Human albumin functions as a general stabilizer during purification, sterile manufacturing and freeze-drying (see review by Wang et al., J. of Parenteral Sci. and Tech. Vol 42, Number 2S, supplement. 1988). Human albumin is also a good cake-former in a formulation for freeze-drying. The use of albumin for stabilization of factor VIII is known and is currently used in all highly purified factor VIII products on the market.
However, it is not desirable to add human albumin to a therapeutic protein manufactured by recombinant DNA technology. In addition, the use of human albumin as a formulation excipient often limits the use of many of the most powerful and sensitive analytical methods for protein characterization.

There is a need for albumin free formulations containing factor VIII and especially recombinant factor VIII which are stable during drying or freeze-drying, in solution and as a solution after reconstitution.

Several solutions have been proposed for the stabilization of different proteins:

EP 35 204 (Cutter) discloses a method for imparting thermal stability to a protein composition in the presence of a polyol.

EP 381 345 (Corint) discloses an aqeous liquid of a peptide, desmopressin, in the presence of carboxymethylcellulose.

In WO 89/09614 (Genentech), a stabilized formulation of human growth hormone comprising glycine, mannitol and a buffer is disclosed and in a preferred embodiment a non-ionic surfactant such as polysorbate 80 is added. The non-ionic surfactant is added for reduced aggregation and denaturation. The formulation has an increased stability in a lyophilized formulation and upon reconstitution.

EP 268 110 (Cetus) discloses a solution comprising a particular protein, interleukin-2, which is dissolved in an inert carrier medium comprising a non-ionic polymeric detergent as a solubilizer/stabilizer. The preferred

detergents are octylphenoxy polyethoxy ethanol compounds, polyethylene glycol monostearate compounds and polyethylene sorbitan fatty acid esters.

US 4 783 441 (Hoechst)discloses an aqueous solution comprising a protein, such as insulin and a surface active substance.

US 4 165 370 (Coval) discloses a gamma globulin solution and a process for the preparation thereof. The solutions contains polyethylene glycol (PEG). A non-ionic surfactant can be added to the solution.

In EP 77 870 (Green Cross) the addition of amino acids, monosaccarides, oligosaccarides or sugar alcohols or hydrocarbon carboxylic acid to improve stability of a solution containing factor VIII is disclosed and the addition of sugar alcohol or disaccarides to an aqueous solution of factor VIII for increasing stability during heat treatment has been disclosed in EP 117 064 (Green Cross).

WO 91/10439 (Octopharma) claims stable injectable solution of factor VIII or factor IX which comprises a disaccaride, preferably saccarose and one or more amino acids.

EP 315 968 and EP 314 095 (Rorer) claim stable formulations of factor VIII with different ionic strength.

Proteins are different with regard to physico-chemical properties. When preparing a pharmaceutical preparation which should be physico-chemical acceptable, and stable for a long time, consideration can not only be taken to the physiological properties of the protein but also other aspects must be considered such as the industrial manufacture, easy handling for the patient and safety for the patient. The results of these aspects are not predictable when testing different formulations and there often is a unique solution for each protein.

In plasma circulating factor VIII is stabilized by association with its carrier protein, the von Willebrand factor (vWF). In plasma and also in conventional intermediate purity factor VIII concentrates the ratio vWF to factor VIII is at least 50:1 on a weight basis. In very high purity factor VIII

concentrates, with a specific activity of more than 2 000 IU per mg protein, the ratio vWF to factor VIII is about 1:1 (w/w) and essentially all factor VIII is bound to vWF. Despite this stabilization further protection by the addition of albumin is required in order to achieve an acceptable stability during lyophilization and storage.

All super pure preparations on the market are stabilized with albumin (human serum albumin).
There is a now a demand for injectable factor VIII without albumin and containing a minimum of additives.

We have now developed a new formulation which solves the above mentioned problems for factor VIII.

To our great surprise we have found that factor VIII, which is a very sensitive protein, can be stabilized without albumin, when a non-ionic surfactant is added.

Thus the present invention relates to a composition comprising a coagulation factor VIII and a non-ionic surfactant as stabilizer. Our factor VIII is highly purified, i.e. has a specific activity of more than 5000 IU/mg protein, and the composition is stabilized without the addition of albumin.
When factor VIII is recombinant it can be either in its full-length form or as a deletion derivative such as SQ derivative.
The amount of factor VIII is from 10 to 100 000 IU/ml, preferably 50 to 10 000 IU/ml.
The non-ionic surfactant is preferably chosen from block co-polymers such as a poloxamer or polyoxyethylene (20) fatty acid ester, such as polysorbate 20 or polysorbate 80. Tween 80® has been used as polysorbate 80.
The non-ionic surfactant should be present in an amount above the critical micelle concentration (CMC). See Wan and Lee, Journal of Pharm Sci, 63, 136, 1974.
The polyoxyethylene (20) fatty acid ester is thus preferably in an amount of at least 0.01 mg/ml. The amount could e.g. be between 0.02 and 1 mg/ml.
The composition can also comprise sodium or potassium chloride, preferably in an amount of more than 0.1 M.

The composition comprises preferably a calcium salt such as calcium chloride or calcium gluconate preferably in an amount of more than 0.5 mM and an amino acid such as L-histidine in an amount of more than 1 mM. The amount could e.g. be chosen between 0.05 and 500 mM. Mono-or disaccarides such as sucrose or sugar alcohols could be added e.g. in an amount of 1 to 300 mg/ml.

The composition comprises preferably L-histidine and sucrose. The ratio sodium chloride to L-histidine in the composition is preferably more than 1:1.

The composition could comprise
i) 10-100 000 IU/ml of recombinant factor VIII
ii) at least 0.01 mg/ml. of a polyoxyethylene (20) fatty acid ester
iii) sodium chloride, preferably in an amount of more than 0.1 M.
iv) calcium salt such as calcium chloride or calcium gluconate preferably in an amount of more than 0.5 mM.
v) an amino acid such as L-histidine in an amount of more than 1 mM.

To this composition could mono-or disaccarides or sugar alcohols, preferably sucrose be added.
The composition could be in a dried form, preferably lyophilized or in aqeous solution before or after drying. The dried product is reconstituted with sterile water for injection or a buffer solution.

The claimed composition can also be a stable aqeous solution ready for use.

The invention also relates ot compositions in which the specific activity of r-VIII SQ is more than 12 000 IU / mg protein , preferably more than 14 000 IU / mg.

The claimed composition can be prepared by mixing factor VIII with a non-ionic surfactant in an aqeous solution, preferably together with an amino acid such as L-histidine, sodium salt, sucrose and a calcium salt or by eluating factor VIII from the last purification step with a buffer containing a non-ionic surfactant in an aqeous solution, preferably together with an amino acid such as L-histidine, sodium salt, sucrose and a calcium salt.

The invention also relates to the use of a non ionic surfactant preferably chosen from block co-polymers, preferably a poloxamer or polyoxyethylene (20) fatty acid ester, preferably polysorbate 20 or polysorbate 80, as stabilizer for a composition comprising coagulation factor VIII.

An amino acid is used to buffer the system and it protects also the protein in the amorphous phase. A suitable buffer could be L-histidine, lysine and/or arginine. L-Histidine has primarily been chosen because of the good buffer capacity of L-histidine around pH 7.

Sucrose or sugar alcohol can also be added for the protection of the protein.

Calcium (or divalent metal ions), here added as calcium chloride ($CaCl_2$) but other salts such as calcium gluconate, calcium glubionate or calcium gluceptate can also be used, is necessary for the maintenance of the association of factor VIII heavy and light chain.

The data presented in the examples indicate that r-VIII SQ is stable for at least 12 months when stored at 5±3°C.

The following examples illustrate the invention and show stability data for different formulations, all falling under the patent protection, a protection which is not limited to these examples.

The following figures are illustrating the invention:

Figure 1 HPLC gelfiltration, Example 10A, stored 5 months at 25°C.
Figure 2 HPLC gelfiltration, Example 10B, stored 5 months at 30°C.

EXPERIMENTAL

Material and methods

The production of recombinant factor VIII SQ (r-VIII SQ) was essentially performed as described in patent WO 91/09122, example 1-3. A DHFR deficient CHO celline (DG44N.Y.) was electroporated with an expression vector containing the r-VIII SQ gene and an expression vector containing the dihydrofolate-reductase gene. Following selection on selective media surviving colonies were amplified through growth in stepwise increasing amounts of methotrexate. Supernatant from the resulting colonies were individually screened for VIII:C activity. A production clone was chosen and this was subsequently adapted to serum free suspension growth in a defined medium and finally a large scale fermentation process was developed. Supernantant is collected after certain time periods and further purified as described below.

The clarified conditioned medium was pH adjusted and applied to a S-Sepharose FF column. After washing, factor VIII was eluated with a salt buffer containing 5 mM $CaCl_2$.

Immunoadsorption was carried out on an immunoaffinity resin where the ligand was a monoclonal antibody (8A4) directed towards the heavy chain of Factor VIII. Before loading to the column the S-eluate was treated with 0,3 % TNBP and 1 % Octoxynol 9.
The column was equilibrated, washed and factor VIII was eluated with a buffer containing 0,05 M $CaCl_2$ and 50 % ethylene glycol.

The mAb-eluate was loaded on a Q-Sepharose FF column, equilibrated with the elution buffer in the immunoaffinity step. After washing, factor VIII was eluated with 0,05 M L-histidine, 4 mM $CaCl_2$, 0,6 M NaCl, pH 6,8.

The Q-eluate was applied to a gel filtration column (Superdex 200 p.g.). Equilibration and elution was carried out with a formulation containing sodium chloride, L-histidine, calcium chloride and polysorbate 80.

The protein peak was collected and the solution was formulated before freeze drying.

The VIII:C activity and the concentration of the inactive components were adjusted by diluting with an appropriate buffer. The solution was then sterile filtered (0,22 µm), dispensed and freeze-dried. Samples from each composition were frozen and stored at - 70 °C. These samples were thawed and used as references during the assay of VIII:C.

The coagulant activity VIII:C was assessed by a chromogenic substrate assay (Coatest Factor VIII, Chromogenix AB, Mölndal, Sweden). Activated factor X (Xa) is generated via the intrinsic pathway where factor VIII:C acts as cofactor. Factor Xa is then determined by the use of a synthetic chromogenic substrate, S-2222 in the presence of a thrombin inhibitor I-2581 to prevent hydrolysis of the substrate by thrombin. The reaction is stopped with acid, and the VIII:C, which is proportional to the release of pNA (para-nitroaniline), is determined photometrically at 450 nm against a reagent blank. The unit of factor VIII:C is expressed in international units (IU) as defined by the current International Concentrate Standard (IS) established by WHO.

The recovery of VIII:C is calculated as the percentage of VIII:C in the reconstituted solution divided by the VIII:C in the frozen and thawed solution for freeze-drying with appropriate adjustment for dilutions.

Soluble aggregates were determined by gel filtration. A prepacked Superdex 200 HR 10/30 column (Pharmacia) was used with a fluoroscence detector (exitation wavelength 280 nm), emission wavelength 340 nm). The reconstituted preparation were analysed. Evaluation of results from gelfiltration was done by visual examination of the chromatograms, or by integration of the peak areas if aggregates were found.

Recovery over freeze drying is expressed in % yield of frozen reference.

Example 1. Comparison between albumin and non-ionic surfactant.

Recombinant factor VIII was prepared according to the method described under Experimental.

Two ml of the solution was lyophilized and thereafter reconstituted in an amount of 5 ml of sterile water for injections.

The compositions were the following :

	1 A	1B	1 C	1D
L-Histidine, mM	50	50	50	50
Sodium chloride, M	0,6	0,6	0,6	0,6
Calcium chloride, mM	4	4	4	4
Polysorbate 80, %	-	-	0.02	-
PEG 4000, %	0.1	0.1	-	-
Albumin, %	-	1	-	1
VIII:C charged IU/ml	250	250	250	250
Recovery, IU/ml after reconstit.	83	197	232	222

This example shows that there was no difference in the recovery of factor VIII:C when the non ionic surfactant or albumin was used.

Example 2, Comparison between different strengths of non ionic surfactant

Recombinant factor VIII was prepared according to the method described under Experimental.

Two ml of the solution was lyophilized and thereafter reconstituted in an amount of 2 ml of sterile water for injections.

The compositions were the following :

	2 A	2B	2 C
L-Histidine/L-Glutamate equimolar amount, mg/ml	10	10	10
Sodium chloride, %	2	2	2
Calcium chloride, mg/ml	0.1	0.1	0.1
Polysorbate 80, %	-	0,001	0,01
VIII:C charged IU/ml	300	300	300
Recovery, IU/ml after reconstit.			
Initial	69	133	228
3.5 h*	43	140	222
7h*	49	133	204

* stored as reconstituted solution at ambient temperature

It is here clearly shown the surprisingly good stabilizing effect on factor VIII when a non ionic surfactant is used.

Example 3 , Variation of non-ionic surfactant concentration.

Recombinant factor VIII was prepared according to the method described under Experimental.

Two ml of the solution was lyophilized and thereafter reconstituted in an amount of 5 ml of sterile water for injections.

	3 A	3B	3 C	3D	3E
L-Histidine, mM	50	50	50	50	50
Sodium chloride, M	0.34	0.34	0.34	0.34	0.34
Calcium chloride, mM	4	4	4	4	4
Polysorbate 80, %	0.01	0.02	0.03	0.04	0.05
Recovery, after reconstit., %	91	90	93	99	100

Results from this example indicate that the recovery of factor VIII (VIII:C) was very high after reconstitution and good for all concentrations of polysorbate 80 used.

Example 4. Variation of sodium chloride concentration

Recombinant factor VIII was prepared according to the method described under Experimental.

Two ml of the solution was lyophilized, stored at different temperatures for up to 6 months (mon) and thereafter reconstituted in an amount of 5 ml of sterile water for injections.

		4 A	4B
L-Histidine.mM		50	50
Sodium chloride, M		0.3	0.6
Calcium chloride, mM		4	4
PEG-4000 %		0.1	0.1
(Polyethylene glycol)			
Polysorbate 80, %		0.025	0.025
Recovery , %, initial		85	86
stored at 8°C			
	3 mon	88	87
	4 mon	87	83
	6 mon	87	83
stored at 25°C,	1 mon	92	93
	3 mon	87	79
	4 mon	84	81
	6 mon	85	85
stored at 37°C	1 mon	88	90
	3 mon	80	80
	4 mon	80	77
	6 mon	81	80
stored at 50°C	1 mon	84	89
	3 mon	77	77
	4 mon	73	70

0,3 or 0,6 M sodium chloride showed very good stability. Both formulations were stable for 6 months at 37°C.

Example 5. Variation of L-Histidine concentration

Recombinant factor VIII was prepared according to the method described under Experimental.

2,2 ml of the solution was lyophilized, stored at different temperatures for up to 3 months (mon) and thereafter reconstituted in an amount of 5 ml of sterile water for injections.

		5 A	5B
L-Histidine, mM		46	59
Sodium chloride, M		0.31	0.31
Calcium chloride, mM		3,7	3,7
PEG-4000 %		0.091	0.091
(Polyethylene glycol)			
Polysorbate 80, %		0.364	0.364
Recovery , %			
stored at 8°C,	Initial	78	84
	3 mon	70	76
stored at 25°C,	1 mon		
	3 mon	69	74
stored at. 37°C	1 mon	76	85
	3 mon	61	48
stored at 50°C	1 mon	60	73
	3 mon	44	48

This example shows that these different amounts of L-histidine does not effect the stability.

Example 6

Recombinant factor VIII was prepared according to the method described under Experimental.

	6A	6B
L-Histidine, mM	65	65
Sodium chloride. M	0.3	0.3
Calcium chloride, mM	4	4
PEG-4000 %	0	0.1
Tween 80, %	0.025	0.025

These solutions were freezed/thawed 1, 5 and 10 times and the recovery was the following:

	IU/ml	IU/ml
cold	298	291
1 freezing	293	293
5	295	287
10	290	288

These studies showed that VIII:C was stable after repeated freeze-thawing and that PEG-4000, which is thought to act as cryoprotectant, is not necessary in this formulation.

Example 7. Variation of pH

Recombinant factor VIII was prepared according to the method described under Experimental.

2,2 ml of the solution was lyophilized and thereafter reconstituted in an amount of 5 ml of sterile water for injections.

	7 A	7B	7 C	7D
L-Histidine, mM	65	65	65	65
Sodium chloride, M	0,3	0,3	0,3	0,3
Calcium chloride, mM	4	4	4	4
Polysorbate 80, %	0.025	0.025	0.025	0.025
pH	6.0	6.5	7.0	7.5
Recovery, %, Initial	74	70	78	79
3 hours*	73	80	78	77

*stored as reconstituted solution at ambient temperature

This example shows that a pH is of no significant importance between 6.0 and 7.5 approx.

Example 8 Addition of sucrose

Recombinant factor VIII was prepared according to the method described under Experimental.

2,2 ml of the solution was lyophilized and thereafter reconstituted in an amount of 5 ml of sterile water for injections.

	8A	8B
L-Histidine, mM	58	20.5
Sodium chloride. M	0.3	0.3
Calcium chloride, mM	3,7	3,7
Sucrose, mM	0	13.3
Polysorbate 80, %	0.025	0.025

Sucrose was added to the solution B after the final purification step before lyophilization.

The recovery after freeze-drying was 76 % for A and 87 % for B. The same activity was found 4 hours after reconstitution stored at room temperature.

This study indicated that the addition of sucrose is favourable for the recovery of VIII:C over freeze-drying.

Example 9 . Variation of calcium salt

Recombinant factor VIII was prepared according to the method described under Experimental.

Two ml of the solution was lyophilized and thereafter reconstituted in an amount of 5 ml of sterile water for injections.

	9 A	9B	9C	9D
L-Histidine, mM	23	23	23	23
Sodium chloride, M	0,34	0,34	0.34	0,34
Calcium chloride, mM	4	4	0.15	0.15
Polysorbate, %	0.025	0.025	0.025	0.025
Sucrose, mM	-	10	-	10
Calciumgluconate, mM	0	0	6	6
Recovery,%, Initial	63	74	74	78
4 hours*	60	73	73	77

*stored as reconstituted solution at ambient temperature

This example shows that $CaCl_2$ can be substituted by Calcium gluconate.

Example 10

Recombinant factor VIII was prepared according to the method described under Experimental.

2,2 ml of the solution was lyophilized and thereafter reconstituted in an amount of 5 ml of sterile water for injections. VIII:C per vial in reconstituted preparation was about 1000 IU.

	10A	10B
L-Histidine, mM	14.7	58
Sodium chloride. M	0.31	0.31
Calcium chloride, mM	3.7	3.7
Sucrose, mM	19.9	-
Polysorbate 80, %	0.025	0.025
Recovery, IU/ml after reconstitution		
Initial	213	198
4 h, 25 °C	213	198
24, 25 °C	201	182
Recovery , %		
Initial	92	91
5 months, 25°C	88	-
5 months, 30°C	76	85
12 months, 7°C	89	97

The recovery was good when part of the L-histidine was substituted by sucrose.

These formulations were studied by gelfiltration after 5 months storage at 25°C and 30°C, respectively and the results are shown in figures 1 and 2. The only peaks to be seen is the peak at 42, indicating factor VIII:C and the peak at 70 which is histidine. Aggregates is to be found earlier than 40. From figure 1 it can be seen that no detectable amount of aggregates was found after 5 months at 25°C for 10A. Figure 2 shows a small amount of aggregates which is less than 2 % after 5 months at 30°C for 10B .

Example 11

Recombinant factor VIII was prepared according to the method described under Experimental.

2,2 ml of the solution was lyophilized and thereafter reconstituted in an amount of 5 ml of sterile water for injections. VIII:C per vial in reconstituted preparation was about 500 IU.

		11A	11B
L-Histidine, mM		14.7	58
Sodium chloride, M		0.31	0.31
Calcium chloride, mM		3.7	3.7
Sucrose, mM		19.9	-
Polysorbate 80, %		0.025	0.025
Recovery, IU/ml after reconstitution			
Initial		98	105
4 h, 25 °C		96	103
24, 25°C		93	101
Recovery , %			
Initial		91	93
stored at 25°C,	5 mon	89	87
stored at 30°C,	5 mon	76	79
stored at 7°C	12 mon	88	89

Both formulations showed good stability.

These formulations were studied by gelfiltration and the results were similar as shown in Figures 1 and 2.

No aggregation was formed when the formulations had been stored for 5 months at 25°C and 30°C, respectively.

Example 12

Recombinant factor VIII was prepared according to the method described under Experimental.

2 ml of the solution was lyophilized, stored at different temperatures for up to 3 months (mon) and thereafter reconstituted in an amount of 4 ml of sterile water for injections. VIII:C per vial in reconstituted preparation was about 500 IU.

	12A	12B
Mannitol, mg/ml	20	20
L-Histidine, mg/ml	2,67	2.67
Sodium chloride, mg/ml	18	18
Calcium chloride, mM	3,7	3,7
Polysorbate 80, mg/ml	0.23	0.23
Recovery , %		
initial	91	93
stored at. 7°C 5 mon	90	85

An acceptable stability was achieved after five months at 7°C.

CLAIMS

1. A composition comprising coagulation factor VIII and a non-ionic surfactant as stabilizer.

2. A composition according to claim 1 in which factor VIII is highly purified and stable without the addition of albumin.

3. A composition according to claim 1 or 2 in which factor VIII is full-length or a deletion derivative of recombinant factor VIII.

4. Composition according to any of claims 1-3 in which the amount of factor VIII is 10 to 100 000 IU/ml, preferably 50 to 10 000 IU/ml.

5. Composition according to any of claims 1-4 in which the non-ionic surfactant is present in an amount above the critical micelle concentration.

6. Composition according to any of claims 1-5 in which the non-ionic surfactant is chosen from block co-polymers, preferably a poloxamer or polyoxyethylene (20) fatty acid ester, preferably polysorbate 20 or polysorbate 80.

7. Composition according to claim 6 in which the polyoxyethylene (20) fatty acid ester is in an amount of at least 0.01 mg/ml.

8. Composition according to any of claims 1-7 which comprises sodium or potassium chloride, preferably in an amount of more than 0.1 M.

9. Composition according to any of claims 1-8 which comprises calcium salt such as calcium chloride or calcium gluconate preferably in an amount of more than 0.5 mM.

10. Composition according to any of claims 1-9 which comprises an amino acid such as L-histidine in an amount of more than 1 mM.

11. Composition according to any of claims 1-10 which comprises mono-or disaccarides, prferably sucrose or sugar alcohols.

12. Composition according to any of claims 10-11 which comprises L-histidine and sucrose.

13. Composition according to claim 8 and 10 in which the ratio sodium chloride to L-histidine is more than 1:1.

14. Composition according to any of claims 1-13, comprising
i) 10-100 000 IU/ml of recombinant factor VIII
ii) at least 0.01 mg/ml. of a polyoxyethylene (20) fatty acid ester
iii) sodium chloride, preferably in an amount of more than 0.1 M.
iv) calcium salt such as calcium chloride or calcium gluconate preferably in an amount of more than 0.5 mM.
v) an amino acid such as L-histidine in an amount of more than 1 mM.

15. Composition according to any of claims 1-14 which is dried.

16. Composition according to claim 15 which is lyophilized.

17. Composition according to any of claims 1-14 which is in a stable aqeous solution ready for use.

18. Composition according to any of claims 3-17 in which the specific activity of r-VIII SQ is more than 12 000 IU / mg protein , preferably more than 14 000 IU / mg.

19. Process for the preparation of the composition according to claim 1 characterized by mixing factor VIII with a non ionic surfactant in an aqueous solution, preferably together with an amino acid such as L-histidine, sodium salt, sucrose and a calcium salt.

20. Process for the preparation of the composition according to claim 1 characterized by eluating factor VIII from the last purification step with a buffer containing a non-ionic surfactant in an aqeous solution, preferably

together with an amino acid such as L-histidine, sodium salt, sucrose and a calcium salt.

21. Use of a non ionic surfactant preferably chosen from block co-polymers, preferably a poloxamer or polyoxyethylene (20) fatty acid ester, preferably polysorbate 20 or polysorbate 80, as stabilizer for a composition comprising coagulation factor VIII.

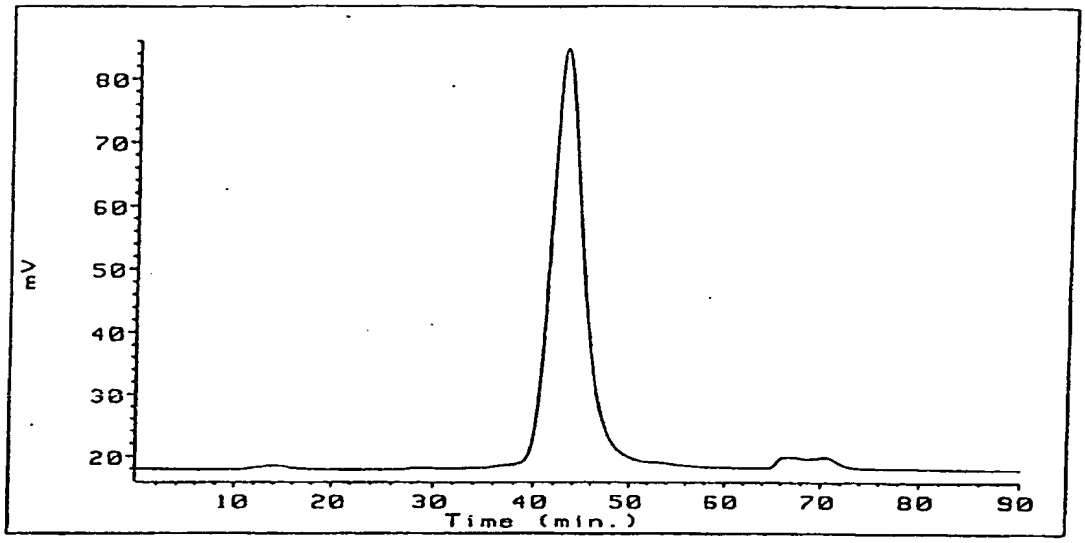

Figure 1

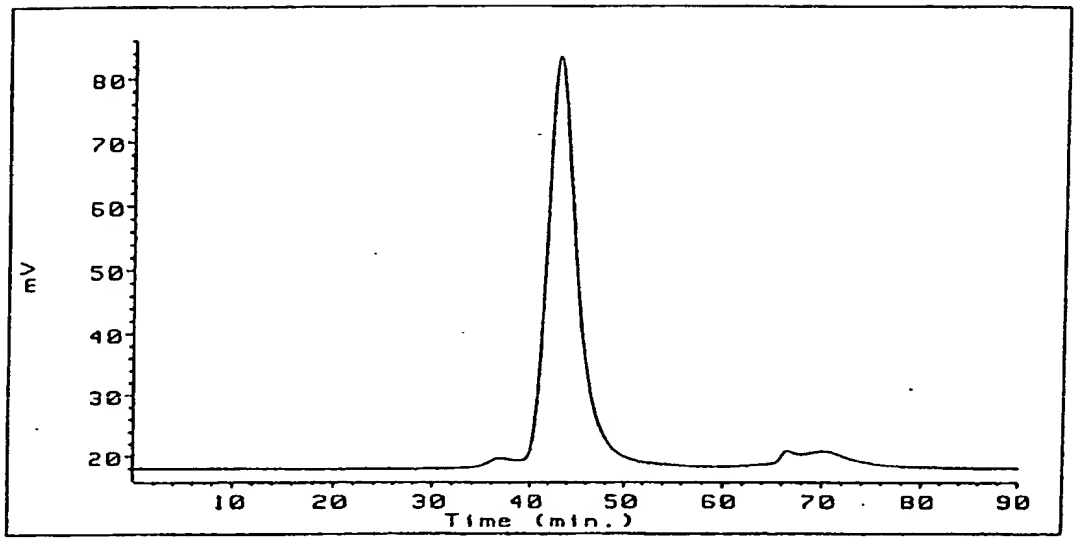

Figure 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 93/00793

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: A61K 35/16, A61K 37/02
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: A61K, C07K, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	EP, A1, 0508194 (BEHRINGWERKE AG), 14 October 1992 (14.10.92), see claim 6, examples 1-2	1-21
X	EP, A3, 0099445 (NEW YORK BLOOD CENTER, INC.), 1 February 1984 (01.02.84), see page 8, line 7 - line 14; page 19, line 24 - page 20, line 27	1-21
A	WO, A1, 9110439 (OCTA PHARMA AG), 25 July 1991 (25.07.91)	1-21

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 January 1994	12 -01- 1994
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Mikael G:son Bergstrand Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

27/11/93

International application No.
PCT/SE 93/00793

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A1- 0508194	14/10/92	AU-A- 1470292	15/10/92
		DE-A- 4111393	15/10/92
		JP-A- 5097702	20/04/93
EP-A3- 0099445	01/02/84	SE-T3- 0099445	
		AU-B- 561900	21/05/87
		AU-A- 1346283	20/10/83
		CA-A- 1207229	08/07/86
		JP-A- 58222023	23/12/83
		US-A- 4481189	06/11/84
		US-A- 4591505	27/05/86
WO-A1- 9110439	25/07/91	DE-A- 4001451	01/08/91
		EP-A- 0511234	04/11/92

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E8

Review

Stability of Protein Pharmaceuticals

Mark C. Manning,[1,2] Kamlesh Patel,[1] and Ronald T. Borchardt[1]

Recombinant DNA technology has now made it possible to produce proteins for pharmaceutical applications. Consequently, proteins produced via biotechnology now comprise a significant portion of the drugs currently under development. Isolation, purification, formulation, and delivery of proteins represent significant challenges to pharmaceutical scientists, as proteins possess unique chemical and physical properties. These properties pose difficult stability problems. A summary of both chemical and physical decomposition pathways for proteins is given. Chemical instability can include proteolysis, deamidation, oxidation, racemization, and β-elimination. Physical instability refers to processes such as aggregation, precipitation, denaturation, and adsorption to surfaces. Current methodology to stabilize proteins is presented, including additives, excipients, chemical modification, and the use of site-directed mutagenesis to produce a more stable protein species.

KEY WORDS: protein stability; biotechnology; mutagenesis; denaturation.

INTRODUCTION

With the recent advances in recombinant DNA technology, the commercial production of proteins for pharmaceutical purposes has become feasible (1,2). As a result, the preparation of proteins as medicinal agents has become an integral part of the pharmaceutical industry. Currently, there are more than 150 recombinant proteins in Phase I clinical trials or beyond, and almost a dozen have received FDA approval. Unfortunately, proteins possess chemical and physical properties which present unique difficulties in the purification, separation, storage, and delivery of these materials. Therefore, formulation of proteins differ greatly from that of rigid small organic molecules. Future pharmaceutical scientists will need to be properly trained to address the various aspects of protein instability. An introduction to these concepts is presented below, with the view that understanding protein stability at a molecular level is essential to solving many of their formulation problems.

Degradation pathways for proteins can be separated into two distinct classes, involving chemical instability and physical instability. First, chemical instability can be defined as any process which involves modification of the protein via bond formation or cleavage, yielding a new chemical entity. Second, physical instability does not involve covalent modification of the protein. Rather, it refers to changes in the higher order structure (secondary and above). These include denaturation, adsorption to surfaces, aggregation, and precipitation. A summary of the current understanding of each of these processes is presented and illustrated by well-characterized systems. Finally, approaches for retarding or inhibiting these processes and, thereby, increasing protein stability is presented.

CHEMICAL INSTABILITY

A variety of chemical reactions is known to affect proteins (Fig. 1). These reactions can involve hydrolysis, including both cleavage of peptide bonds as well as deamidation of Asn and Gln side chains.[3] Hydrolysis at Asp-X sites is particularly accelerated. Oxidation of Cys can lead to disulfide bond formation and exchange, whereas oxidation of Met and other amino acids may inactivate or alter the activity of a protein. Other decomposition reactions include beta-elimination and racemization.

Deamidation

In the deamidation reaction, the side chain amide linkage in a Gln or Asn residue is hydrolyzed to form a free carboxylic acid. Over the past two decades many investigators have observed altered forms of proteins which have been attributed to deamidation. Such a list contains lysozyme (3), bovine growth hormone (bGH) (growth hormone is also known as somatotropin) (4), human growth hormone (hGH) (5,6), insulin (7,8), α-crystallin (9), cytochrome c (10), γ-immunoglobulin (11), epidermal growth factor (EGF) (12), hemoglobin (13), triosephosphate isomerase (TIM) (14,15), neocarzinostatin (16), prolactin (17), gastrin releasing peptide (18), and adrenocorticotropic hormone (ACTH) (19,20), suggesting that *in vitro* deamidation is a common phenomenon.

The hydrolysis of Asn and Gln residues for many proteins and peptides has been observed under a variety of

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[3] Unless otherwise noted, all amino acids listed are L-enantiomers of the 20 common amino acids and are referred to by their three-letter abbreviations.

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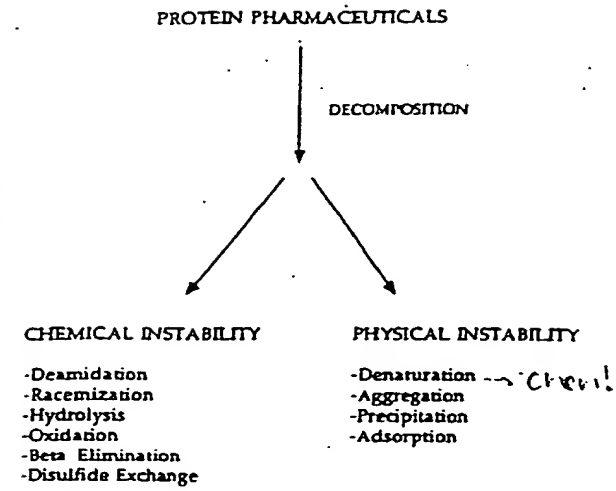


Fig. 1. Summary of the chemical and physical instability processes observed in protein pharmaceuticals.

chemical conditions and has been reviewed by Robinson and Rudd (21). Interestingly, it was realized that the deamidation of Asn residues, which occurs most often at the sequence Asn-Gly, was accelerated at neutral or alkaline conditions (22–24). The rates were also higher relative to the hydrolysis of the amino acid Asn itself (21). An explanation is that deamidation is believed to proceed through a five-membered cyclic imide intermediate formed by intramolecular attack of the succeeding peptide nitrogen at the side chain carbonyl carbon of the Asn residue (see Fig. 2) (25). Subsequently, the cyclic imide spontaneously hydrolyzes to give a mixture of peptides in which the polypeptide backbone is attached via an α-carboxyl linkage (Asp) or is attached via a β-carboxyl linkage (iso-Asp) (23). Similarly, Gln can also undergo deamidation via formation of a six membered ring (23). Most of the information on the mechanism and rate of deamidation of

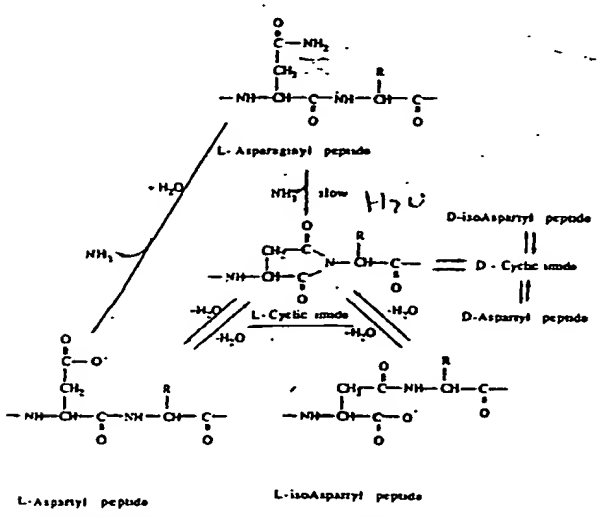


Fig. 2. Pathways for spontaneous deamidation, isomerization, and racemization for aspartyl and asparaginyl peptides.

Asn residues has been obtained from studies on short model peptides (26,27). Clear evidence for a deamidation mechanism involving the cyclic imide intermediate has been obtained by Geiger and Clarke (26). In their study, deamidation of a hexapeptide sequence based on residues 22–27 of ACTH (Val-Tyr-Pro-Asn-Gly-Ala) was studied at 37°C and pH 7.4. Evidence from these studies supporting cyclic imide formation include the appearance of iso-Asp, Asp, and cyclic imide peptides upon deamidation and a ratio of the iso-Asp to Asp peptide formed in the deamidation of this hexapeptide (2.8:1) is the same as that found when purified cyclic imide is hydrolyzed (3.1:1). If there is a significant amount of direct solvent hydrolysis of the amide linkage occurring, the proportion of Asp peptide relative to iso-Asp peptide in the deamidation of a hexapeptide would have increased, which is not the case. The presence of iso-Asp products from the incubations of proteins and peptides implies cyclic imide formation as an intermediate in deamidation reaction (28–32). The Fourier transform infrared photoacoustic spectroscopic measurements (FTIR-PAS) have also provided direct evidence for the formation of a cyclic imide in peptides with Asn-Gly sequences induced by heating in the dry state (33).

Recently, we have shown that both Asp- and iso-Asp-hexapeptides are formed upon deamidation of Val-Tyr-Pro-Asn-Gly-Ala, ACTH$^{22-27}$ (Asn-hexapeptide), in the pH range of 5 to 12 at 37°C (34,35). This further confirms the formation of a cyclic imide intermediate in the deamidation process at neutral and alkaline pH's. In the pH range 7 to 12, buffer concentration had significant effect on the rate of deamidation, indicating general acid-base catalysis. No buffer catalysis was observed at pH 5, 6, and 6.5. The ratio of iso-Asp- and Asp-hexapeptides was independent of buffer concentration at all pH's and was approximately 4:1. At acidic pH's (pH 1–2), the deamidation was much slower than at alkaline pH, and only Asp-hexapeptide was produced upon deamidation. Although iso-Asp-hexapeptide was not detected at acidic pH, one new product (Val-Tyr-Pro-Asp) was observed by HPLC. These results suggest that at acidic pH, the probable mechanism of deamidation is direct hydrolysis of the amide side chain of Asn, to form the Asp-hexapeptide, which further degrades in acidic media via peptide bond cleavage at the Asp-Gly bond. Reactions at pH 3 and 4 were very slow at 37°C (degradation of Asn-hexapeptide was not detected for 60 days).

By comparison, when deamidation experiments were carried out with ACTH (1–39), the separation of Asp- and iso-Asp products could not be achieved by either isoelectric focusing or cation-exchange HPLC (20). However, these techniques did separate native ACTH from the deamidated ACTHs (Asp- and iso-Asp-ACTH). The rate constants for the deamidation of both ACTH and Asn-hexapeptide (ACTH$^{22-27}$) at pH 2.0, 7.0, and 9.6 at 37°C were similar. Formation of the iso-Asp product upon deamidation of ACTH at pH 7.0 and 9.6 was verified by the protein carboxymethyltransferase (PCM)-catalyzed methylation of deamidated ACTH. No such methylation was observed when ACTH was incubated at pH 2.0, 37°C. These data indicate the involvement of cyclic imide intermediate at neutral and alkaline pH but not at pH 2.0.

Since the formation of a cyclic imide involves participation of the succeeding amino acid, the size and physico-

chemical properties of neighboring amino acid side chain is expected to play an important role in the rate of formation of cyclic imide. Evidence in support of this conclusion comes from studying the rates of cyclic imide formation in peptides containing Asn (26) or Asp β-benzyl esters (36–45). For example, the rate of cyclic imide formation at pH 7.4 was approximately 50 times slower in the Asn-Leu-hexapeptide than the Asn-Gly-hexapeptide due to steric hinderance by the Leu side chain (26).

Robinson and co-workers (46–49) investigated the nonenzymatic deamidation of Asn residues in synthetic pentapeptides, and the effects of amino acid sequence, pH, temperature, buffer species, and ionic strength. Using synthetic pentapeptides, it has been shown that deamidation is favored by increased pH, temperature, and ionic strength (46,47). These studies showed the importance of primary sequence around the Asn residue, but did not investigate the formation of either iso-Asp or cyclic imide. Similar results are obtained for cytochrome c (50). Similarly, the rate of deamidation of human TIM was facilitated by high temperatures, and was also found to be dependent on the presence of substrate and specific buffers (14). Unlike the hydrolysis of peptides containing esters of Asp, where the cyclic imide intermediate can be trapped (22,24), cyclic imide formation during the deamidation of Asn peptides is the rate-determining step (26,27).

The rates of deamidation of Asn residues in proteins are influenced by the secondary and tertiary structures of proteins. Clarke has shown that Asp and Asn residues in native proteins generally exist in conformations where the peptide bond nitrogen atom cannot approach the side chain carbonyl carbon without large scale conformational changes (51). Therefore, certain proteins will not undergo deamidation unless they have been denatured. Cyclic imide could only be formed *in vitro* at Asn^{67} of bovine pancreatic ribonuclease in the unfolded state (52). While in the native structure, this residue is poorly positioned for cyclic imide formation. Similarly, it has been shown that urea (a strong denaturant) accelerates the deamidation of bGH, hGH, and prolactin, presumably by unfolding the protein (5). Tertiary structure appears to be the principle determinant for the deamidation of trypsin (53). The study also showed that adjacent Ser residues aid in the formation of the cyclic imide intermediate, consistent with earlier studies on small peptide systems (36,37). Recently, Lura and Schirch (54) have shown that the mechanism of deamidation of Val-Asn-Gly-Ala and N-acetyl-Val-Asn-Gly-Ala varies according to the conformation of the peptide backbone. Above pH 9.0, both peptides have similar conformations and thus deamidate by the same mechanism to give mixture of Asp and iso-Asp peptides. However, at pH 7.0, while the N-acetyl peptide yielded a mixture of Asp and iso-Asp peptides, the non-acetylated peptide gave no detectable amounts of these products, but rather yielded a cyclic peptide believed to be formed by nucleophilic attack of the amide of the Asn residue by the terminal amino group.

It is well known that peptides of Asp esters undergo intramolecular cyclization, under both acidic and basic conditions, leading to a cyclic imide derivative (39,55). However, no reports are available showing the formation of cyclic imide from Asn peptides in acidic media. There are few examples in the literature which at least indicate that cyclic imide is not involved in the deamidation reaction under acidic conditions. For example, insulin (7,8), neocarzinostatin (16), and ribonuclease A (56), when incubated in acidic media, yield only Asp-containing products from the deamidation of Asn residues. Similar results were obtained by Meinwald and co-workers (27), where Ac-Asn-Gly-NHMe produced only Ac-Asp-Gly-NHMe and the analogous iso-Asn produced only the iso-Asp-containing peptide after 1 day in 1 *M* HCl.

It has been postulated that deamidation may play a central role as a timer in protein turnover and in aging (21). However, for pharmaceutical preparations, the major concern is the change in protein function upon deamidation. In a few cases, the deamidation of specific Asn residues has been linked to the changes in the protein function, for example, deamidation at two Asn-Gly sequences in TIM resulted in subunit dissociation (15). Deamidation at an Asn-Gly site in a hemoglobin mutant (Hb providence) changed its oxygen affinity (57), and deamidation at an Asn-Asp site in hGH altered its proteolytic cleavage properties (58). Recently, deamidation was shown as one of the major chemical processes responsible for irreversible enzyme inactivation of lysozyme (59) and ribonuclease (60) at 100°C. Deamidation was also responsible for the decrease in biological activity for porcine ACTH (62) and slower rate of refolding after deamidation for ribonuclease (63,64).

With small peptides, the iso-Asp and Asp peptides are separable by chromatographic or electrophoretic methods (65–67). However, with larger proteins similar methodology has not been successful. Chromatofocusing (68) and HPLC (20,29) have been used for separating the native protein from the product mixture, but these techniques do not separate the iso-Asp-peptide from the Asp-peptide. However, there are several indirect ways of showing the presence of iso-Asp residues in proteins. These include (i) NMR methods to distinguish Asp and iso-Asp (27,54,69); (ii) Leu aminopeptidase digestion, since this enzyme will not cleave an iso-Asp peptide bond (67,70); (iii) tryptic peptide mapping and amino acid sequencing (71); and (iv) use of PCM, which is known to methylate selectively the free α-carboxy group of iso-Asp peptides (72). Recently, Johnson and co-workers have shown the use of this enzyme as a powerful analytical tool for estimating minimum levels of protein deamidation (73). In their work, they monitored the increase in methylation for aldolase, bovine serum albumin, cytochrome c, lysozyme, ovalbumin, ribonuclease A, and TIM upon incubation at pH 11, finding evidence that iso-Asp is formed upon deamidation.

Oxidation

The side chains of His, Met, Cys, Trp, and Tyr residues in proteins are potential oxidation sites. Even atmospheric oxygen can oxidize Met residues. Oxidation has been observed in many peptide hormones during their isolation (74–77), synthesis (78), and storage (79). Since the thioether group of Met is a weak nucleophile and is not protonated at low pH, it can be selectively oxidized by certain reagents under acidic conditions (80). For example, hydrogen peroxide can modify indole, sulfhydryl, disulfide, imidazole, phe-

nol and thioether groups of proteins at neutral or slightly alkaline conditions, but under acidic conditions the primary reaction is the oxidation of Met to Met sulfoxide (81). In addition to hydrogen peroxide, a variety of other reagents have been used to oxidize Met to Met sulfoxide. These include periodate, iodine, dimethylsulfoxide, a dye-sensitized photooxidation, chloramine-T, and *N*-chlorosuccinamide (82,83). To oxidize Met to Met sulfone, more drastic conditions and reagents are needed, e.g., 95% performic acid. The structures of the oxidation products of Met, i.e. Met sulfoxide and Met sulfone, are shown in Fig. 3.

Oxidation of Met residues to their corresponding sulfoxides is associated with loss of biological activity for many peptide hormones [e.g., corticotropin (84), α- and β-melanotropins (85), parathyroid hormone (86), gastrin (87), calcitonin (88), and corticotropin releasing factor (77)] as well as nonhormonal peptides and proteins (81). It has been shown that *E. coli* ribosomal protein L12 loses activity after oxidation of Met residues to Met sulfoxide and that the activity can be restored by incubating the protein with high concentrations of β-mercaptoethanol (89). Restoration of biological activity was found to coincide with the reduction of Met sulfoxide to Met (89). Alpha-1-proteinase inhibitor protein, which is a major serum inhibitor of elastase activity, loses its ability to inactivate elastase when chemically oxidized (90,91). Oxidation by hydrogen peroxide of a single Met residue in subtilisin at pH 8.8 occurs concurrently with changes in kinetic parameters of the enzyme, although it does not abolish enzymatic activity (92). Similar results were obtained with a disulfoxide derivative of α-chymotrypsin (93,94), and trypsin (95). In many cases, such as parathyroid hormone (86), ribonuclease S-peptide (96), ribonuclease (97), and lysozyme (98), reduction of Met sulfoxide by thiols results in the recovery of nearly full biological activity.

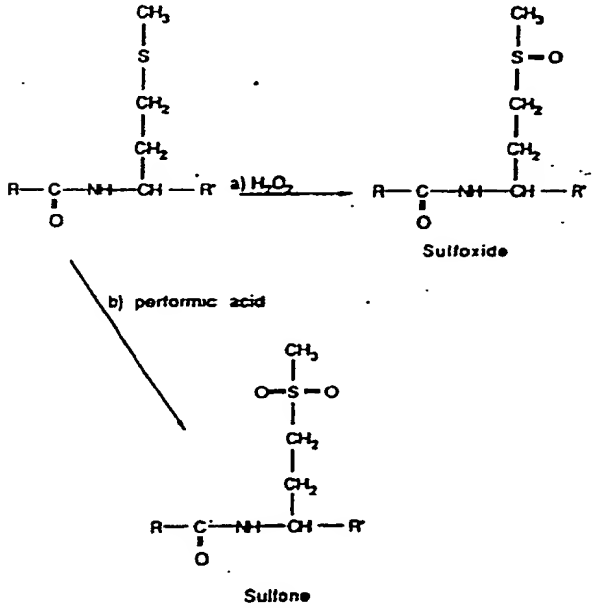


Fig. 3. Mechanism of oxidation of Met-containing peptide under (a) mild and (b) strong conditions.

There are also examples where protein functions are not affected upon Met oxidation. Active monosulfoxide derivatives of pancreatic ribonuclease (99), α-chymotrypsin (100), and Kunitz trypsin inhibitor (101) have been prepared using mild hydrogen peroxide treatment at low pH (pH 1 to 3). Similarly, EGF (102,103) and glucagon (104) are biologically active when chemically oxidized.

It is also shown that within a given protein, the reactivity of Met residues towards oxidation may be different depending upon their position. For example, in hGH, Met^{170} was found to be completely resistant to oxidation by hydrogen peroxide (105). In addition, it was shown that when biosynthetic hGH is chemically oxidized at Met^{14}, it exhibits full biological activity and has immunoreactivity identical to that of authentic hGH (6). In human chorionic somatomammotropin (hCS), Met^{64}, Met^{16}, and Met^{179} have markedly different reaction rates (105). The oxidation of Met^{64} and/or Met^{179} markedly reduced both its affinity for lactogenic receptors and its *in vitro* biological potency (105).

Determination of oxidized Met in proteins is generally a problem, because during conventional amino acid analysis Met sulfoxide is converted to Met during acid hydrolysis. Therefore, Met is commonly determined by using its specific reactions with alkyl halides (106) or cyanogen bromide (107), to which the sulfoxide is resistant. After alkylating the Met residues of the peptide, its Met sulfoxide is oxidized with performic acid to the acid stable sulfones; the sulfone content, determined by amino acid analysis, is then used to correct the Met estimate obtained by conventional amino acid analysis (99). Alternatively, Met containing peptides have been separated from peptides containing oxidized Met residues by ion-exchange chromatography (108), countercurrent distribution (109), HPLC (103,110), or affinity chromatography (111). A radioassay for non oxidized Met in peptide hormones based on its specific reaction with iodo[2-^{14}C]acetic acid is also developed (112).

The thiol group of Cys (RSH) can be oxidized in steps, successively, to a sulfenic acid (RSOH), a disulfide (RSSH), a sulfinic acid (RSO_2H), and, finally, a sulfonic acid (RSO_3H), depending upon reaction conditions. The factors which influence the rate of oxidation include the temperature, pH, and buffer medium used, the type of catalyst (e.g., traces of metal ions), and the oxygen tension (113). An important factor is the spatial positioning of the thiol groups in the proteins. In those cases where contact between thiol groups within the molecule of the protein is hindered, or when the protein contains only a single thiol group, intramolecular disulfide bonds are not formed, but sometimes, under favorable steric conditions, intermolecular disulfide bonds arise, and the protein aggregates (114). Thiol groups are oxidized not only when oxidizing agents (e.g., iodine, ferricyanide, tetrathionate, *O*-iodosobenzoate, and hydrogen peroxide) are added, but also "spontaneously," by oxygen from the air (autooxidation). The oxidation of thiol groups by molecular oxygen takes place at an appreciable rate in the presence of catalytic quantities of metal ions, such as iron and copper ions (115,116). The speed of oxidation of thiol groups is also greatly influenced by the nature of neighboring

groups. This was clearly demonstrated by Barron *et al.* (117) and also by Ovaberger and Ferraro (118). From their findings it appears that the rate of oxidation of dithiols is diminished on increasing the distance between the thiol groups in the molecule and also under the influence of neighboring electronegative groups such as carboxyl group (i.e., groups that raise the pK_a of the thiol group). This fact indicates that the mercaptide ion is oxidized more easily than the undissociated thiol group. Thus, it is shown that usually the oxidation rate increases with increasing pH (119). At 90°C and pH 8.0, α-amylase from *Bacillus* was shown to undergo irreversible thermoinactivation due to air oxidation of the Cys residues along with formation of incorrect or "misfolded" structures (120). Inactivation of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase by hydrogen peroxide has been shown to result from sulfhydryl group modification to sulfenic acid (114). Various methods for quantitative determination of thiol and disulfide groups in proteins are described by Torchinskii (121).

The side chains of His, Tyr, Met, Cys, and Trp residues can also be oxidized by visible light in the presence of dyes, i.e., via photooxidation. The specificity for the various amino acid side chains is particularly determined by pH. Oxidation of His is a rapid reaction at neutral pH but is quite slow at low pH. At higher pH, Tyr is most reactive (122–124), while Trp and Met are the only amino acids readily oxidized below pH 4. More information on photooxidation is available in a few review articles (125–127). In many cases, loss of enzymatic activity following photooxidation has been attributed to the destruction of critical His residues. For example, the inactivation of rabbit muscle aldolase (128), pig heart aspartate aminotransferase (129,130), cytochrome c (131), renin, and yeast enolase (132) has been attributed to photodegradation of His residues.

Proteolysis

It has been established that peptide bonds of Asp residues are cleaved in dilute acid at a rate at least 100 times faster than other peptide bonds (133). Selective hydrolysis is usually achieved by heating for 5–18 hr at 110°C in either 0.03 *N* HCl or 0.25 *N* acetic acid (134). The mechanism of hydrolysis undoubtedly involves intramolecular catalysis by a carboxyl group of the Asp residue. Hydrolysis can take place at either the N-terminal and/or C-terminal peptide bonds adjacent to the Asp residue. Inglis (135) has described the mechanism for such hydrolysis as shown in Fig. 4, where cleavage of the N-terminal peptide bond would proceed via an intermediate containing a six membered ring rather than via a five-membered ring as proposed for C-terminal peptide fission. Such peptide bond cleavage can contribute to the inactivation of proteins. Significant irreversible thermoinactivation in lysozyme (59) and ribonuclease A (60) at 90–100°C and pH 4 was found to be due to peptide bond cleavage at Asp-X bonds.

It is now well established that Asp-Pro peptide bonds are particularly labile and are hydrolyzed under conditions where other Asp peptide bonds are stable (136). For example, when rabbit antibody light chain was subjected to 10% acetic acid-pyridine (pH 2.5) in 7 *M* guanidinium hydrochloride (GnHCl) for 24–90 hr, selective cleavage was observed at Asp^{109}-Pro^{110} (137). Piszkiewicz *et al.* have suggested that the hydrolytic reaction proceeds via intramolecular catalysis by carboxylate anion displacement of the protonated nitrogen of the peptide bond and the rate enhancement occurs due to the greater basicity of the Pro nitrogen (136). Marcus has compared the lability of the Asp-Pro bonds to the lability of other peptide bonds, in particular to those of Asp residues (138). In his study, a variety of dipeptides was heated at 110°C in 0.015 *M* HCl. The concentration of amino acid released during the heat treatment was determined by amino acid analysis. The results indicated that Asp-Pro bonds were 8- to 20-fold more labile than other Asp-X or X-Asp peptide bonds. Other peptide bonds that do not involve Asp were found to be stable to hydrolysis under these conditions.

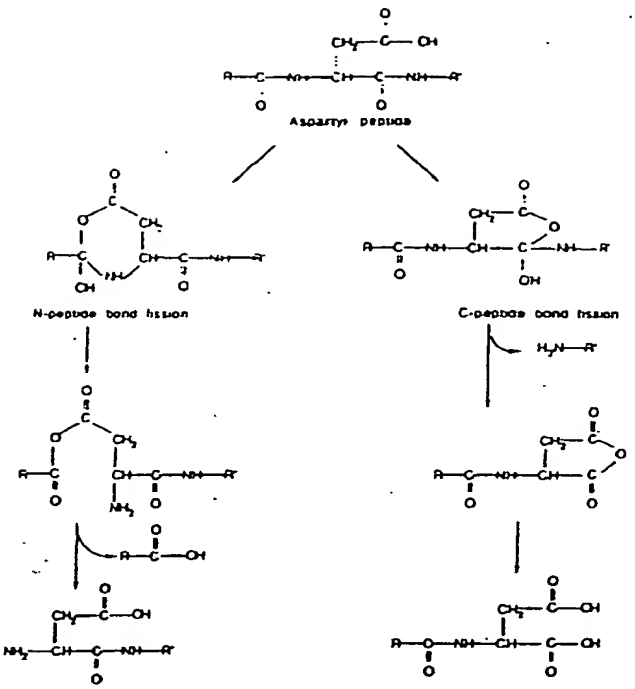


Fig. 4. Mechanism of degradation of aspartyl peptides in acidic media.

Asp-X peptide bonds also undergo a reversible isomerization between the Asp and the iso-Asp forms via the cyclic imide intermediate as shown in Fig. 3 (139,140). This reaction was first noted by Swallow and Abraham with Asp-Lys derived from hydrolyzates of bacitracin (139). Similar interconversion was also shown for Val-Tyr-Pro-Asp-Gly-Ala ($ACTH^{22-27}$), displaying a half-life of 53 days at pH 7.4 and 37°C (26). Even storage of aqueous solutions of an Asp-containing peptide can result in the formation of cyclic imide derivatives (141). The ring closure is particularly fast when an Asp residue is followed by Gly in the sequence (142). Peptide bonds formed by X-Ser and X-Thr are also labile, but require strong acidic conditions (e.g., 11.6 *M* HCl) (143). The mechanism involves N–O acyl rearrangement (144).

The time course of hydrolysis of amide peptide bonds

can be monitored by gel chromatography or sodium dodecylsulfate polyacrylamide gel electrophoresis (145), and quantitatively assessed by gel scanning densitometry (146). The identities of the amino acids at the new carboxyl and amino termini resulting from peptide chain hydrolysis can be determined by hydrazinolysis/HPLC (147) and dansylation/TLC (148), respectively. Identification of the position of the new termini in the sequences of the protein can be learned from amino acid analysis by HPLC of the sequential digest of the COOH terminus by carboxypeptidases (149) and the NH_2 terminus by the Edman degradation procedure (150,151). Recently, a radioassay was used to study peptide bond hydrolysis at neutral pH and room temperature (152). In this work, a peptide (Phe-Phe-Phe-Gly), radiolabeled with ^{14}C at the α carbon of the C-terminal residue, was attached to resin and the release of radiolabel due to amide bond hydrolysis was monitored. The half life for this peptide at neutral pH was found to be 7 years (152).

Incorrect Disulfide Formation

Sulfhydryl groups and disulfide bonds and their interrelationships are an important factor affecting the properties of the majority of proteins. The interchange of disulfide bonds can result in incorrect pairings, leading to an altered three-dimensional structure and, hence, loss of catalytic activity. The reaction mechanism is different in alkaline and acidic media (153,154). In neutral and alkaline media the reaction is catalyzed by thiols, which, in the form of thiolate ions, carry out nucleophilic attack on a sulfur atom of the disulfide (Fig. 5). Catalytic quantities of thiols can arise by hydrolytic cleavage of disulfides to carry out such disulfide exchange. For example, lysozyme, when heated at 100°C at neutral pH, undergoes beta-elimination of Cys to produce free thiols, which cause disulfide interchange (155). Benesch and Benesch have studied the mechanism of disulfide exchange in acidic media and they proposed that the exchange takes place through a sulfenium cation, which is formed by attack of a proton on the disulfide bond (156). The sulfenium cation carries out an electrophilic displacement on a sulfur atom of the disulfide (Fig. 6). Addition of thiols can inhibit such exchange by scavenging the sulfenium cations.

Disulfide exchange can be prevented if thiol scavengers, such as *p*-mercuribenzoate, *N*-ethylmaleimide, or copper ion, which catalyzes the air oxidation of thiols, are present (157,158). Zale and Klibanov recently reported that the irreversible loss of activity of ribonuclease A at 90°C and pH 8 was significantly lower when incubated in the presence of above-mentioned reagents (60). Proteins with scrambled disulfide bonds can be rearranged to yield native, catalytically active material by incubating the protein with small amounts of thiols, such as mercaptoethanol or Cys (159,160).

Racemization

All amino acid residues except Gly are chiral at the carbon bearing the side chain and are subject to base catalyzed racemization. Racemization is generally considered to proceed through the removal of the α-methine hydrogen by base to form a carbanion (see Fig. 7) (161). Stabilization of this carbanion controls the rate of recemization. Racemization of amino acids in protein can generate non-metabolizable forms of amino acids (D-enantiomers) or create peptide bonds inaccessible to proteolytic enzymes.

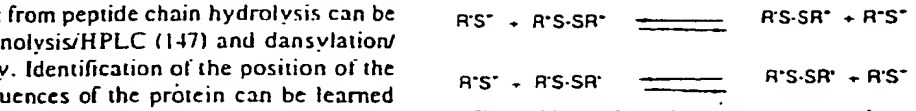
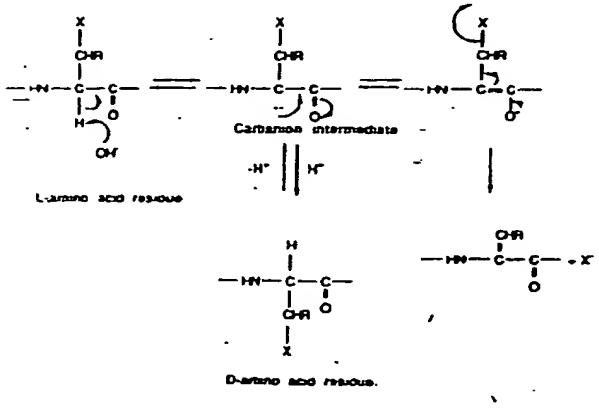


Fig. 5. Mechanism of disulfide exchange in neutral and alkaline media.

Fig. 6. Mechanism of disulfide exchange in acidic media.

The relative rates of racemization of amino acid residues in 37 dipeptides was studied at pH 7.5 at 123°C by Smith and Desol (162). They found that the relative rates of racemization of amino acid residues in dipeptides is determined by a delicate balance of factors, including inductive and field effects, intramolecular base action, intramolecular solvation, and steric hindrance to solvation. Racemization of Asp is particularly interesting, because it is shown that Asp residues in proteins racemizes 10^5-fold faster than the free amino acid (163), in contrast to a 2- to 4-fold increase for all other residues. An explanation for this exceptional behaviour of Asp is contained in the mechanism of its racemization. The mechanism involves the formation of a cyclic imide through nucleophilic attack on the β-carbonyl carbon by the α-nitrogen of the succeeding amino acid (Fig. 2). Resonance structures of the cyclicimide ring involve charge transfer between nitrogen and not only the α-carbonyl but also the β-carbonyl group. The latter resonance allows the peptide carbonyl to intensify resonance with the α-carbanion, formed by proton abstraction as an intermediate in the racemization process (164). Racemization of Asp was shown



X = H, OH, O-glycosyl, O-phosphoryl, SH, SCH_2-R, aliphatic or aromatic residue

R = H or CH_3

Fig. 7. Mechanism of beta-elimination and racemization reactions in alkaline media.

to proceed via cyclic imide at pH 7.4 and 37°C for ACTH$^{22-27}$ with a half life of 19.5 hr (26).

Beta Elimination

High-temperature inactivation of proteins often results from the destruction of disulfide bonds (59,155,158). Chemically, this is the result of β-elimination from the cystine residue. Furthermore, this can occur at lower temperatures at high pH (165,166). Whether this is a general decomposition pathway for protein pharmaceuticals is still unknown, but Volkin and Klibanov have studied more than a dozen proteins at 100°C and found that they all undergo β-elimination of disulfides at similar rates (155). As with previous findings, the rates were greatly accelerated under alkaline conditions. Therefore, under conditions which may lead to rapid deamidation, other chemical instability may also occur. Finally, the resultant thiols from the elimination reaction will certainly contribute to other degradation pathways (aggregation, adsorption, precipitation). Overall, the introduction of disulfides in an effort to increase stability may not always be effective (vide infra).

Other amino acid residues can also undergo β-elimination. For example, Cys, Ser, Thr, Phe, and Lys can be degraded via β-elimination at alkaline conditions as shown in Fig. 7. In many cases, the β-elimination reaction is influenced by pH, temperature and presence of metal ions. For example, the initial rates of β-elimination in phosvitin (167), antifreeze glycoprotein (168), and lysozyme (169) were directly proportional to hydroxide ion concentration and also shown to depend on temperature, while the rate of β-elimination of phosphoserine in phosvitin was markedly enhanced by the addition of calcium chloride (167).

PHYSICAL INSTABILITY

Physical instability is a difficulty rarely encountered for small molecule drugs. However, proteins, because of their polymeric nature and their ability to adopt some form of superstructure (secondary, tertiary, quaternary), can undergo a variety of structural changes independent of chemical modification. Globular proteins fold in a manner in which exposure of hydrophobic groups is minimized (170–174). Loss of globular structure, that is, loss of tertiary structure, is referred to as denaturation. It is possible for a protein to become denatured and still retain some secondary structure (175–188). Once unfolded, the polypeptide chain can undergo further inactivation by association with surfaces (adsorption), aggregation with other protein molecules, or some chemical reaction. Should aggregation lead to macroscopic ensembles, this process is termed precipitation. While the exact interrelationship between all of these pathways is still unclear, each is well documented. Generally, it is believed that denaturation must first occur for other physical instability processes (adsorption, aggregation, precipitation) to proceed.

Denaturation

Denaturation refers to an alteration of the global fold of a molecule, that is, a disruption of the tertiary and, frequently, secondary structure (172,188–195). Often, denaturation is equated with protein instability, and many review articles refer just to this aspect (189–195). Nevertheless, it is the most widely studied facet of protein inactivation (170,172,189–201). Caused by a variety of conditions (increase in temperature, decrease in temperature, extreme pH, addition of organic solvents or other denaturants), this process can be envisioned as reversible or irreversible. These terms can be misleading, as, in some instances, native structure (and subsequently, activity) can be recovered from irreversibly denatured proteins. Reversible denaturation is defined as unfolding caused by an increase in temperature which can be reversed by subsequent lowering of the temperature. Irreversible denaturation is any unfolding process which does not allow the native structure to be regained simply by lowering the temperature. Although a protein which is irreversibly denatured may still be returned to its native state by addition of denaturant followed by dialysis, the process is still defined as irreversible.

Usually, unfolding is thought to be the cooperative transition between the native (N) and unfolded (U) states of a protein [see Eq. (1)].

$$N \rightleftharpoons U \tag{1}$$

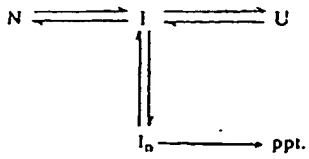
For such a two-state system, an increase in temperature will cause a rapid change in structure from N to U at the melting temperature, T_m, where T_m is defined as the temperature at which 50% of the molecules are unfolded (i.e., $\Delta G = 0$). An increase in T_m is indicative of a more stable protein structure, even though, strictly speaking, stability should be defined as the Gibbs free energy at a given temperature. It should be noted that T_m can be quite dependent on pH and concentration. For example, the T_m of T4 lysozyme is 42°C at pH 2 and 65°C at pH 6.5 (202,203). Enzymes, such as T4 lysozyme, are attractive systems for study as their activity as well as structure can be assayed (190). In any case, recovery of activity or structure upon lowering the temperature is considered reversible denaturation.

Irreversible denaturation or inactivation actually refers to a variety of processes. Such proteins may be simply "misfolded," that is, in a conformation which does not allow them to renature properly (204–206), or they may have undergone some additional process, whether chemical or physical. The ability to recover activity by the addition of denaturants (e.g., GnHCl or urea), followed by dialysis, indicates a structural (aggregation, adsorption, etc.) or misfolding component to the irreversible inactivation.

For mutants of T4 lysozyme, the irreversible denaturation of the protein proceeds through different pathways (202). For the wild type, the inactivation appears to be conformational in nature; whereas the activity of mutants containing a disulfide bridge (e.g., Ile^3 → Cys-Cys^{97}/Cys^{54} → Val) could not be recovered by the addition of GnHCl. Chemical inactivation was postulated to be the mechanism involved. Interestingly, this seems to be unrelated to the effect of the disulfide bridge on the reversible denaturation of T4 lysozyme. Although less unfolded, mutants containing the disulfide bridge are more susceptible to chemical inactivation. This opposes the usual behavior of proteins, in which they are more likely to undergo a chemical reaction when unfolded (192,207,208).

Aggregation

The existence of partially unfolded intermediates (Scheme I) has now been shown to exist for many proteins (175,178–183,186–188,209,210). While they may be important in protein folding, their role in protein stability is less evident. However, for bGH and interferon-γ (If-γ), these intermediates may lead to inactivation via aggregation. The following scheme (Scheme I) has been proposed for both bGH (182) and If-γ (178).



Scheme I. Model of folding phases for proteins which display partially unfolded intermediates.

Moderate amounts of denaturant can generate a partially unfolded intermediate of bGH (180,186,188), whose solubility is less than either the N or the U states of bGH. This species, I, associates or aggregates, as outlined in Scheme I. Retaining much of the native secondary structure, the tertiary structure of I is mostly lost. The site of interaction has been identified as an amphiphilic helix (residues 107–128) which is part of the central four-helix bundle (180,182,183). Increasing the amphiphilicity of this region via mutagenesis produces a protein which associates more readily, precipitates faster, and renatures more slowly. Renaturation is hindered, since the associated intermediate, I_n, is not directly along the pathway for return to the native conformation.

Interferon-γ is inactivated by acid treatment (209,211). Below pH 4.5 and in the absence of NaCl, the dimeric native state is converted into monomers, which are partially denatured (209). Dialysis does lead to the formation of N, but also to large aggregates (I_n), which have substantially lower activity (175). By circular dichroism spectroscopy (CD), the aggregates retain a large amount of secondary structure, but the tertiary structure is largely disrupted. Addition of salt also leads to formation of I_n (178,210). Interferon-γ is highly positively charged at pH 4.5, and presumably, the chloride ions mask the charge enough to allow the monomers to associate. This behavior is similar to the salt effects which allow the formation of molten globule-like states in acid- and base-denatured proteins (184,185).

Upon denaturation with GnHCl, antithrombin displays a biphasic denaturation, with midpoints at 0.8 and 2.8 *M* GnHCl concentrations (as determined by CD and activity measurements) (179). Therefore, at 1.5 *M*, there is a partially unfolded intermediate, I, which aggregates slowly. Freshly prepared I can be returned to the native state, N, by dialysis. However, once aggregation occurs, the native state cannot be reformed by this approach.

Surface Adsorption

Adhesion of proteins to surfaces is a well-known phenomenon in the field of biomaterials, as biocompatibility is essential for artificial limbs, organs, or even contact lenses (212,213). Interaction of plasma proteins with various surfaces has received the most attention. However, while surface adsorption is potentially disastrous in many facets of protein manipulations, reported studies have been fairly limited. One system which has been well studied is insulin. Primarily, this is because it is the one protein which has been on the market for a long period of time and has been the target of numerous delivery devices. Major difficulties have been encountered with insulin adsorbing to the surfaces of delivery pumps (214–218), to glass and plastic containers (219–223), and to the inside of intravenous bags (224–226).

Precipitation

Precipitation is the macroscopic equivalent of aggregation. Precipitation of proteins has been known to occur for a long time, usually in conjunction with denaturation (195,227,228), but detailed studies of it have been mostly limited to the case of insulin.

Insulin frosting is the formation of a finely divided precipitate on the walls of the insulin container. The proclivity of insulin to precipitate is well established and is particularly troublesome when the insulin is loaded into a long-term infusion device (214–216). Recent work on insulin frosting has shown that the process is accelerated by the presence of a large headspace within the vial (221). Presumably, the insulin is undergoing denaturation at the air–water interface, facilitating the precipitation process. Other factors which can contribute to insulin frosting are the concentration of zinc ion (known to regulate the aggregation state of insulin), pH, and the presence or absence of additives (221). Changes in the types of vials or stoppers did affect the process. Other studies have demonstrated a dependence of insulin precipitation upon the type of materials in contact with the insulin solution (219,222). However, these studies were concerned with much longer time courses (30–120 days).

Upon expression in recombinant organisms, many proteins fail to remain in a soluble form, and often aggregate into macroscopic ensembles termed inclusion bodies (IBs) (229–234). Formation of IBs appears to be a general phenomenon and independent of the degree of overexpression. Thus, it may not simply be a precipitation process. However, the mechanism of IB formation is unknown, and even basic characterization of IBs is lacking (235,236).

Typically, IBs are believed to be comprised of partially or completely denatured protein (232), but evidence is lacking. For proteins containing Cys residues, the possibility of intramolecular disulfide bonds exists. It has been shown that the addition of reducing agents, such as thiols, does aid in the solubilization of IBs (237–239). However, the cytoplasm of *E. coli* is quite reducing, making disulfide formation *in vivo* quite unlikely (240). Consequently, formation of disulfides may be an artifact of IB isolation procedures or the result of localized oxidizing environments (231).

IMPROVING PROTEIN STABILITY

Additives

Additives can be defined as any excipient which is introduced into the formulation in an effort to increase stabil-

ity. Some of the simplest and most effective are salts of other ionic compounds. Salts decrease reversible denaturation via nonspecific binding to the protein (241–247). Specific ion binding sites are also known to exist, even for nonmetalloproteins. Binding of ions to these sites increases thermal stability and has been demonstrated for subtilisin (248–250), ribonuclease (251), thermolysin (252,253), parvalbumin (254), acyl carrier protein (255), alkaline phosphatase (256,257), calbindin D_{9k} (258), copper-zinc superoxide dismutase (259,260), and α-lactalbumin (261–263). Since such a relatively simple process as ion binding can provide a definite increase in thermal stability (i.e., reduced tendency toward denaturation), ion binding sites have been designed into proteins via mutagenesis (see below). In fact, such sites have been constructed in subtilisin (248,250), even without intending to do so. Ion binding can be employed to control physical instability phenomena such as aggregation and precipitation. This has been demonstrated in the case of insulin (264).

Polyalcohol materials, such as glycerol and sugars, are well known to stabilize proteins with respect to denaturation (265–272). Detailed studies by Timasheff and co-workers have established that this occurs through selective solvation of the protein (265–271). At low concentrations of the additive, more water molecules pack around the protein, in order to exclude the more hydrophobic additive. This results in increased stability. At higher concentrations, this is no longer possible, and the more hydrophobic organic solvent begins to denature the protein.

Detergents have often been employed as additives for the stabilization of proteins, including both nonionic (219,222,226) and anionic (273–278) species. Studies on anionic detergents, such as sodium dodecyl sulfate (SDS), have focused on its role in the denaturation of proteins (273–275,277,278). It has also been found to affect the deamidation of food proteins (276). Nonionic detergents, such as Tween and Pluronic, have been evaluated for their ability to prevent adsorption of proteins to surfaces (226,279), to inhibit aggregation and precipitation (219,222), and to hinder denaturation (280,281). These types of additives have the additional advantage of facilitating the delivery of proteins transdermally (282) and intranasally (283).

Site-Directed Mutagenesis

Site-directed mutagenesis refers to the methods that provide the ability to make amino acid substitutions at specific sites in a protein. Therefore, it is now possible to alter the primary sequence of a protein in an effort to increase the overall stability (284,285). Certainly, chemical stability can be increased by replacing susceptible functional groups. However, how amino acid substitutions can affect an increase in thermal stability is still uncertain. A number of approaches have been reported, such as attempts

(i) to improve interior interactions, leading to an increase in thermal stability;
(ii) to increase α helix stability via manipulation of the helix dipole;
(iii) to introduce disulfide bridges in order to stabilize the native conformation and provide stability against reversible thermal denaturation;
(iv) to design ion binding sites which will also increase thermal stability; and
(v) to replace potential sites for chemical degradation, such as deamidation.

(i) Stabilization via Increased Efficiency in Packing. While the overall relationship between amino acid substitutions and stability is not yet clear, some basic principles are emerging. Decreasing conformational flexibility, improving hydrophobic packing, and maximizing hydrogen bonding should all increase stability. Due to its lack of a side chain, Gly possesses the most conformational freedom of all the naturally occurring amino acids. Replacement with Ala should restrict this freedom, thereby decreasing the entropy of unfolding and producing increased stability. This has been demonstrated in two systems. In λ repressor, the substitution of Ala for Gly (mutants $Gly^{46} \rightarrow Ala$ and $Gly^{48} \rightarrow Ala$) produces a 0.7 to 0.9 kcal/mol increase in stability (286). This relates to an increase in T_m of 3–5°C. Other substitutions at position 48 (Ser, Asn) also provided increased stability but less than with Ala. The double mutant is more stable by 1.1 kcal/mol. Lack of additivity to the stability of the protein suggests that the conformation for the double mutant has been altered from that of the wild type (287). Similar results have been observed for T4 lysozyme (288), where the $Gly^{77} \rightarrow Ala$ mutant is more stable by 0.4 kcal/mol. Conformational flexibility can be further reduced by introduction of Pro into the polypeptide chain. Identifying an Ala residue with φ and ψ angles (289) which could accommodate replacement with Pro, the $Ala^{82} \rightarrow Pro$ mutant was found to be 0.8 kcal/mol more stable.

Hydrophobic interactions are believed to be important for stability of globular proteins and play a role as nucleation sites in protein folding (170,172,290,291). In barnase, Ile^{96} is part of a cluster of hydrophobic amino acids. Replacement with Val destabilizes the protein toward reversible denaturation by 1.2 kcal/mol, whereas substitution with Ala produces a 4.0 kcal/mol decrease (292). Such extensive destabilization does not correlate simply to differences in hydrophobicity. Meanwhile, mutations involving Ile^3 in T4 lysozyme do produce stability changes which correlate with changes in hydrophobicity of the side chain (293).

Hydrogen bonding is certainly an important force in the stabilization of protein structures. Mutagenesis has been used to determine the effect of hydrogen bonding in protein stability. In particular, the hydrogen bond between the side chain of Thr^{157} and the amide proton of residue 159 of T4 lysozyme was examined in detail (294). Amino acids which could not accept a hydrogen bond from the amide destabilized the structure. Crystal structures of the mutants indicated that the degree of disruption of the structure did not correlate to the observed instability, suggesting that the structural basis for stability is still not completely understood.

Mutagenesis can be done in a random fashion, as well as in a site-specific manner (284,295–301). For T4 lysozyme, many of the mutants were found to be destabilizing (302,303) or had no effect (304) with respect to reversible denaturation. An investigation of Trp to Tyr mutants found that the $Trp^{138} \rightarrow Tyr$ mutant had a T_m 3°C lower than the wild type, whereas the triple mutant ($Trp^{126} \rightarrow Tyr/Trp^{138} \rightarrow Tyr/Trp^{158} \rightarrow Tyr$), having every Trp replaced by Tyr, was less

stable by 7°C at pH 2.0 (296). In contrast, the activity and structure (as determined by CD) were comparable for all three proteins. Crystallographic characterization of various mutants of T4 lysozyme (305) lead to the conclusion that the differences in stability arose from effects on the folded state, and not on the unfolded state (305,306).

Random mutations in λ repressor produced little change in stability. Of 12 mutants, 10 showed identical T_m values to the wild type, while one was lower and one was greater (297). In staphylococcal nuclease (STN), stability was measured by resistance to GnHCl and urea denaturation (298). The mutants seemed to fall into one of two classes. The most interesting were those that displayed greater stability than the wild type at temperatures above 55°C but less than the wild type at 20°C (termed Class I). Unlike in the T4 lysozyme system, the effects were interpreted in terms of their effects on the unfolded state. This was later substantiated by CD studies on large STN fragments (299). Class I mutations produced fragments with a greater degree of structure then the wild type at intermediate concentrations of denaturant.

(ii) Stabilization of the Helix Dipole. The α helix conformation allows the dipoles of the individual peptide groups to add in a constructive manner (307–310), producing a significant dipole moment for extended helices. The helix dipole has been implicated as playing a role in protein folding (310) and the stability of superstructures such as the four-helix bundle (311,312), a common structural motif (313).

Employing the C- and S-peptide fragments from ribonuclease as a model, Baldwin and co-workers have systematically evaluated α helix stability, particularly with regard to the effect of the helix dipole (314–318). While hydrophobic interactions and salt bridges also play a role (315,316,318–321), the helix dipole appears to be the primary factor involved in helix stability. Reinforcement of the helix dipole is achieved by placing acidic groups near the N-terminus and basic groups near the C-terminus. These principles have now been applied toward stabilization of helices within proteins (300,322,323).

Mutations have been made in T4 lysozyme with the intent to increase the helix dipole and thereby affect an increase in stability (300). Four of the eleven α helices in this protein have no apparent acidic group near its N-terminus and were evaluated as target sites for mutagenesis. Two such single mutants ($Ser^{38} \rightarrow Asp$ and $Asn^{144} \rightarrow Asp$) and the corresponding double mutant were constructed. Both single mutants showed an increase in T_m of 2°C, while the double mutant was approximately 4°C higher. This corresponds to an increase in the free energy of stabilization ($\Delta\Delta G$) of −1.6 kcal/mol for each mutation (300). The additivity of the effects on stability has been observed previously (193, 202,324).

Similarly, the interaction of the protonated form of His^{18} in barnase (a ribonuclease from *Bacillus amyloliquefaciens*) with its α helix provides ~2 kcal/mol of stabilization relative to various mutants (323). In addition, the importance of charge-helix dipole interactions could be directly assessed by varying the pH so that His^{18} was deprotonated, permitting these effects to be studied in the absence of other structural factors introduced by amino acid substitutions.

Replacement of Glu^{34} in helix 3 of λ repressor with a Lys residue produced a 2°C drop in T_m (325). The Glu resides at position 2 of the helix, near the N-terminal end. Removing an acidic group and replacing it with a basic one is in opposition to the helix dipole, thus leading to a decrease in thermal stability.

(iii) Stabilization via Introduction of a Disulfide Group. A common approach toward stabilization of a protein is to introduce a disulfide bond, anticipating that this modification would decrease the entropy of unfolding and increase the tendency toward reversible denaturation. In addition, it was hoped that it would produce an unfolded form which would be less susceptible to irreversible inactivation. Disulfides have been introduced into dihydrofolate reductase (326), subtilisin (327,328), λ repressor (329), and T4 lysozyme (202,330–333). Stability in the subtilisin system has been evaluated by the ability to prevent autolytic degradation. Two possible sites were chosen which appeared to be suitable for the placement of an unstrained disulfide bridge. Both the $Ser^{24} \rightarrow Cys/Ser^{87} \rightarrow Cys$ and the $Thr^{22} \rightarrow Cys/Ser^{87} \rightarrow Cys$ mutants displayed fasted rates of autolysis than the wild type (327), with $Thr^{22} \rightarrow Cys/Ser^{87} \rightarrow Cys$ being much less stable. Investigations into the effects on reversible denaturation were prevented by aggregation difficulties. However, there are data to suggest that there is a correlation between thermal stability and proteolytic susceptibility (327,328), a phenomenon that has been observed previously (198,297,334). As a final note, it has been suggested that the effects of the single Cys mutations will indicate whether the disulfide will be able to stabilize the protein (327). For example, the $Thr^{22} \rightarrow Cys$ mutation so destabilizes T4 lysozyme that even including it in a disulfide bond will not return the mutant protein to the stability level of the wild type.

Formation of a disulfide bond was engineered into *E. coli* dihydrofolate reductase by replacing Pro^{39} with Cys and coupling it to Cys^{85} ($Pro^{39} \rightarrow Cys/Cys^{85}$) (326). Loss of stability was observed, as T_m decreased by 3°C. However, denaturation by GnHCl required a higher concentration to achieve 50% denaturation of the mutant, suggesting that the mutant is more stable. Final analysis of the data did conclude that the mutant was less stable at 25°C than the wild type (284).

Introduction of a disulfide bond into T4 lysozyme also produces differing effects on stability (202,330–333). While the disulfide increase resistance to reversible denaturation, the impact on irreversible denaturation is more complicated (202). Despite being less unfolded at high temperatures, the disulfide-containing mutant is more susceptible to chemical inactivation than the wild type (202), as its activity cannot be recovered by treatment with denaturants. The wild type, while losing activity more rapidly, can be nearly completely reactivated by denaturation with GnHCl followed by dialysis. Again, the effects of mutations may have a quite different impact on reversible and irreversible denaturation of a protein.

(iv) Stabilization via Introduction of Ion Binding Sites. As mentioned above, the binding of ions tends to increase the thermal stability of proteins, whether the interaction is specific or not. Engineering such a site into a protein is believed to be relatively simple, compared to designing some other type of stabilizing interaction (251). In fact, secondary calcium binding sites for subtilisin have been produced inadvertently (248,250). Mutagenesis has been employed to

probe the binding of calcium directly at the binding site (258) and at a distance (249). Eliminating a negative charge in the coordination sphere (by substituting Gln for Glu or Asn for Asp) costs approximately 2 kcal/mol in energy (258). For electrostatic interactions over larger distances (5–20 Å), the effect is more modest (248,249) but still can improve stability (248).

(v) *Stabilization by Removing Chemically Susceptible Sites*. Recently, a unique stabilization strategy was reported for triosephosphate isomerase (335). It involved the replacement of potential deamidation sites, Asn^{14} and Asn^{78}, by Thr and Ile, respectively, producing a genetically engineered protein which was much more stable towards heat inactivation than the native form of the enzyme. Similar results were obtained with recombinant derived human interleukin-1α, where Asn^{36} was replaced with Ser (336). This mutant protein was stabilized against base-catalyzed and temperature-induced deamidation.

Chemical Modification of Proteins

While reactive sites can be removed by mutagenesis, that may not always be possible, if a product has already been identified and produced by recombinant techniques. An alternative is to block such groups with chemical agents. One common, though nonspecific, approach has been to couple peptides and proteins to polyethylene glycol (PEG) (337–339) or poly(oxyethylene) (340,341). While these modifications may increase stability, their effect on conformation, activity, and immunogenicity may be quite dramatic or even undesirable. A more promising derivatization is the attachment of a lipid group to the protein or peptide (342–346), a posttranslational modification which occurs *in vivo* (344–346) and appears to facilitate the insertion of the protein into the lipid bilayer. For insulin modified in this manner, the activity is largely retained (342) and delivery problems may be circumvented (343).

Modification of basic residues in proteins is known to increase thermal stability (347–350). Conversion of Lys to homoarginine via guanidination has been shown to stabilize numerous proteins (347–349), although such treatment did not affect the stability of ribonuclease (350). Methylation of basic residues has been observed in heat-shocked prokaryotes (351). Presumably, the methylated amino acids aid in producing a more thermally stable set of proteins.

Replacement of Met by mutagenesis in order to prevent oxidation has been demonstrated. The logical choice for substitution would be the nonnaturally occurring norleucine (Nle), which has a methylene group in place of the sulfur atom. This amino acid has been shown to possess many of the same properties as Met (352). Koide *et al.* have grown recombinant organisms on Nle-enriched medium deficient in Met, leading to the production of EGF with Nle substituted into the single Met site, thus preventing any possible oxidative decomposition (353).

CONCLUSIONS

Protein instability encompasses many complicated and interrelated chemical and physical processes. Any of these can occur during the production, isolation, purification, analysis, delivery, and storage of protein pharmaceuticals. Many of these reactions appear to be ubiquitous, and, therefore, of concern to the pharmaceutical scientist. Deamidation of Asn, oxidation of Met, and interchange of cystine and Cys are relatively rapid reactions and have been observed in a number of compounds. Fortunately, many of the chemical reactions can be retarded or halted by appropriate choice of conditions, yet, many require only mild conditions to proceed.

Physical instability refers to processes in which no change in the chemical nature of the protein occurs. These include denaturation, aggregation, precipitation, and adsorption to surfaces. While the last three have been observed with small organic drug agents, denaturation is unique to this class of compounds. Indeed, it is implicated in all of the other physical phenomenon and influences the chemical susceptibility of proteins as well. While all of the decomposition reactions listed in this article truly define protein instability, it is resistance to denaturation which is commonly equated with protein stability. Since denaturation can be caused by heating, cooling, freezing, denaturants, pH extremes, and organic solvents, proteins are obviously sensitive to solution conditions, requiring proteins to be stored and shipped as solid materials.

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(54) COMPOSITION CONTAINING SOLUBLE THROMBOMODULINS

(57) The invention relates to a soluble thrombomodulin composition comprising one or more molecular species of soluble thrombomodulins and at least one member selected from the group consisting of maltose, lactose, sucrose, arginine and salts thereof, and nonionic surfactants; a process for producing the composition; a stabilizer and a stabilizing method for soluble thrombomodulins; and an adsorption inhibitor and an adsorption inhibitory method for soluble thrombomodulins. The invention can provide a lyophilized soluble thrombomodulin preparation which is excellent in long-term stability, prevented from being adsorbed onto a container, and useful as a preventive and remedy for diseases related to blood coagulation, and a process for producing the preparation.

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Description

FIELD OF THE INVENTION

This invention relates to a composition containing as its critical components at least one species of soluble thrombomodulin and at least one member selected from maltose, lactose, sucrose, and arginine and a salt thereof; and a method for producing such composition.

This invention also relates to a composition containing as its critical components at least one species of soluble thrombomodulin and a nonionic surface-active agent; and a method for producing such composition.

Furthermore, this invention also relates to a composition containing as its critical components at least one species of soluble thrombomodulin; at least one member selected from maltose, lactose, sucrose, and arginine and a salt thereof; and a nonionic surface-active agent; and a method for producing such composition.

Still further, this invention relates to a stabilizing agent for a soluble thrombomodulin containing at least one member selected from maltose, lactose, sucrose, and arginine and a salt thereof.

Still further, this invention relates to a method for stabilizing a soluble thrombomodulin comprising the step of adding at least one member selected from maltose, lactose, sucrose, and arginine and a salt thereof to the soluble thrombomodulin.

Still further, this invention relates to an anti-adsorption agent for a soluble thrombomodulin containing a nonionic surface-active agent.

Still further, this invention relates to a method for preventing adsorption of a soluble thrombomodulin comprising the step of adding a nonionic surface-active agent to the soluble thrombomodulin.

BACKGROUND ART

Thrombomodulin is a protein found at vascular endothelial cell surface that has a unique nature of converting thrombin from a coagulant enzyme to an anti-coagulant enzyme, and it was reported in 1981 (Esmon et al., Proc. Natl. Acad. Sci. USA, 78, 2249-2254, 1981). In the subsequent report, Esmon et al. reported that they have succeeded in isolating the thrombomodulin from rabbit lung tissue (Esmon et al., J. Biol. Chem., 257(2), 859-864, 1982). The entire DNA sequence and the amino acid sequence of human thrombomodulin were then reported (EMBO J., 6, 1891-1897, 1987; Biochemistry, 26(14), 4350-4357, 1987), and various studies have been conducted to reveal the functions of different domains of the thrombomodulin. Today, it is conceived that thrombomodulin binds with thrombin to form a thrombin-thrombomodulin complex, and the blood coagulation activity of the thrombin is thereby lost; and in turn, the resulting thrombin-thrombomodulin complex activates protein C to induce anti-coagulation activity. In other words, the thrombomodulin may simultaneously induce the blood coagulation inhibitory action and the fibrinolytic action, and clinical application of thrombomodulin is highly awaited.

Conventional therapeutic agents that have been used for diseases related to blood coagulation activity disorders include agents having an anti-coagulation activity such as antithrombin III and heparin as well as agents having a thrombolytic activity such as urokinase, streptokinase, and tissue plasminogen activator. These agents, however, suffer from side effects such as tendency to hemorrhages, and their actions are either inclined to blood coagulation or thrombolysis. In view of such situation, a great expectation is held for the clinical application of a substance that may simultaneously have the anti-coagulation activity and the thrombolytic activity such as thrombomodulin, and a thrombomodulin-like substance that may have the thrombomodulin activity, i.e. both the affinity for thrombin and the protein C-activating activity.

Human thrombomodulin has a low solubility, and when the human thrombomodulin is used for a medicament, such low solubility results in the difficulty of purification as well as the difficulty in producing the preparation. More illustratively, thrombomodulin is a membrane-bound protein comprising five domains, that is, amino terminal domain, domain of EGF-like structure, domain of O-glycosilation site, transmembrane domain, and cytoplasmic domain, and the thrombomodulin of full length amino acid sequence would require a solubilizing agent upon its purification or production into a preparation. Therefore, there have been a strong demand for a thrombomodulin-like substance that may have an increased solubility (which is hereinafter referred to as a soluble thrombomodulin). In consideration of antigenicity and other safety requirements, a natural human thrombomodulin, such as natural human urine thrombomodulin is most desirable. Of the soluble thrombomodulins, typical genetically engineered soluble thrombomodulins are thrombomodulins having transmembrane and cytoplasmic domains deleted therefrom such as Japanese Patent Application Laid-Open Nos. 1(1989)-6219, 2(1990)-255699, 3(1991)-133380, 3(1991)-259084, and 4(1992)-210700; PCT Application Japanese Language Laid-Open Nos. 3(1991)-503757 and 4(1992)-505554, EP474273, WO91/04276, WO91/05803, WO91/15514, WO92/00325, WO92/03149, and WO93/15755; and Doi et al., the Pharmaceutical Society of Japan, 113rd Meeting, Lecture Summaries 3, Lecture No. 30EM14-1, 1993. Typical natural thrombomodulins include human urine soluble thrombomodulins as disclosed in Japanese Patent Application Laid-Open Nos. 63(1988)-30423, 63(1988)-146898, 3(1991)-86900, and 3(1991)-218399; Ishii et al., J. Clin. Invest., 76, 2178-2181, 1985; Hiramoto et al., the Pharmaceutical Society of Japan,

108th Meeting, Lecture Summaries, Lecture No. 6F05 11-1, 1988; Yatani et al., Vessels (Ketsueki to Myakukan), 20, 197-200, 1989; and Yamamoto et al., J. Biochem., 113, 433-440, 1993.

With regard to the genetically engeneered soluble thrombomodulins, Japanese Patent Application Laid-Open No. 1(1989)-6219 discloses a soluble thrombomodulin comprising at least the amino acid sequence of from 345th to 462nd amino acid residues from the amino terminal; Japanese Patent Application Laid-Open No. 2(1990)-255699 discloses a soluble thrombomodulin comprising 115 amino acid residues; Japanese Patent Application Laid-Open No. 3(1991)-133380 discloses a soluble thrombomodulin comprising at least the amino acid sequence of from 1st to 497th amino acid residues from the amino terminal; Japanese Patent Application Laid-Open No. 3(1991)-259084 discloses a soluble thrombomodulin comprising 468 amino acid residues; Japanese Patent Application Laid-Open No. 4(1992)-210700 discloses a soluble thrombomodulin that is not modified with sulfated glycosaminoglican; PCT Application Japanese Language Laid-Open No. 3(1991)-503757 discloses a soluble thrombomodulin that may contain a part of the amino acid sequence of human tissue plasminogen activator; PCT Application Japanese Language Laid-Open No. 4(1992)-505554 discloses a soluble thrombomodulin that may contain a part of amino acid sequence of human tissue plasminogen activator; EP474273 discloses a soluble thrombomodulin containing the thrombin-binding site comprising 19 amino acid residues and the protein C-activation site; WO91/04276 discloses a soluble thrombomodulin having a sugar chain containing chondroitin and/or chondroitin sulfate; WO91/05803 discloses a soluble thrombomodulin that is modified with sulfated glycosaminoglican; WO91/15514 discloses a soluble thrombomodulin wherein methionine is substituted with another amino acid to prevent oxidation. WO92/00325 discloses a recombinant human urine soluble thrombomodulin and mutants thereof; WO92/03149 discloses a soluble thrombomodulin wherein sugar chain at the domain of O-glycosilation site is modified, and a soluble thrombomodulin having the domain of O-glycosilation site deleted therefrom; WO93/15755 discloses a soluble thrombomodulin wherein the amino acid sequence is modified to prevent proteolysis by a proteolytic enzyme; and WO93/25675 discloses a soluble thrombomodulin wherein cofactor activity is modified by modifying the amino acid sequence. Doi et al. discloses a soluble thrombomodulin having added thereto an amino acid sequence containing an acidic amino acid sequence from bovine thrombomodulin (the Pharmaceutical Society of Japan, 113rd Meeting, Lecture Summaries 3, Lecture No. 30EM14-1, p128, 1993).

With regard to natural soluble thrombomodulins from human urine, Japanese Patent Application Laid-Open No. 63(1988)-30423 discloses a mixture of soluble thrombomodulins having molecular weights under non-reduced condition of 200,000, 48,000 and 40,000, respectively; Japanese Patent Application Laid-Open No. 63(1988)-146898 discloses soluble thrombomodulins having molecular weights under non-reduced condition of 39,000 ± 10,000 and 31,000 ± 10,000; Japanese Patent Application Laid-Open No. 3(1991)-86900 discloses soluble thrombomodulins having molecular weights under non-reduced condition of from 55,000 to 58,000 and from 60,000 to 65,000; and Japanese Patent Application Laid-Open No. 3(1991)-218399 discloses soluble thrombomodulins having molecular weights under non-reduced condition of 72,000 ± 3,000 and 79,000 ± 3,000. Ishii et al. discloses soluble thrombomodulins in plasma and urine (J. Clin. Invest., 76, 2178-2181, 1985); Hiramoto et al. discloses several soluble thrombomodulins in blood and urine (the Pharmaceutical Society of Japan, 108th Meeting, Lecture Summaries, Lecture No. 6F05 11-1, 1988); Yatani et al. discloses a soluble thrombomodulin having a molecular weight under reduced condition of 63,000 (Vessels (Ketsueki to Myakukan), 20, 197-200, 1989); and Yamamoto et al. discloses a soluble thrombomodulin comprising 468 amino acid residues (J. Biochem., 113, 433-440, 1993).

In spite of the advantageous high solubility, the soluble thrombomodulin is insufficient in its chemical stability. For example, even if the soluble thrombomodulin is lyophilized, it still suffers from diminished activity and formation of aggregates after prolonged storage at room temperature of several months to several years. Depending on the conditions of the lyophilization, the soluble thrombomodulin may also become slightly denatured. If the soluble thrombomodulin were denatured, and the denatured soluble thrombomodulin with the aggregates formed through such denaturing were administered to human blood, there would be a fair risk of the aggregates, which is a denatured protein, inducing hypersensitivity and other immunological responses as well as thrombosis. At present, even if the soluble thrombomodulin were to be used for a medicine, it is quite difficult to produce a preparation that can be reliably stored for a prolonged period of time without losing the quality required in the medical field.

Several reports are present that disclose particular types of sugars to be capable of stabilizing particular types of proteins. At the same time, some reports disclose that particular types of sugars are incapable of stabilizing particular types of proteins, or that particular types of sugars would destabilize particular types of proteins. For example, destabilization of tubulin by sucrose is disclosed in Biochem. Bhiophys. Acta., 532, 155-160, 1978; and Japanese Patent Application Laid-Open No. 59(1984)-59625 discloses that sugars such as glucose was capable of stabilizing the activity of tumor necrosis factor, while sugars such as lactose, maltose and sucrose were utterly incapable of stabilizing the activity of the tumor necrosis factor.

Production of preparations of thrombomodulin or thrombomodulin-like substances has scarcely been reported. In the "Detailed Description of the Invention" of Japanese Patent Application Laid-Open Nos. 1(1989)-6219 and 2(1990)-255699 and WO91/04276, there is an indication of the use in an injection of sucrose, glycerin, methylcellulose or carboxymethylcellulose as an additive for the purpose of increasing the viscosity of the injection. These references, however, are utterly silent about the stabilizing effect, and moreover, there is no illustrative demonstration for the stabilizing effect.

Japanese Patent Application Laid-Open Nos. 1(1989)-6219, 2(1990)-255699 and 3(1991)-218399 and WO92/00325 describe formulations of the thrombomodulin wherein albumin, purified gelatin or mannitol is added. However, there is no description of the nature or the stability of the formulation not to mention the merit of the addition of such additives.

The inventors of the present invention have prepared a series of compositions containing the human urine soluble thrombomodulin together with albumin, purified gelatin, glycine, glucose, or mannitol, and evaluated the resulting compositions for their stability. However, such compositions failed to exhibit sufficient long term stability. As described above, no technique has been so far disclosed that would enable long term storage at room temperature of the soluble thrombomodulin preparation.

Specific activity of the soluble thrombomodulin is quite high. Accordingly, when the soluble thrombomodulin is clinically used, it is used at a quite minute dose, and it is often diluted to a very low concentration with an infusion for the purpose of continuous administration. It has been found that the soluble thrombomodulin that has been diluted to a low concentration with an infusion is likely to become adsorbed on the surface of the container such as a glass container, a plastic container, and an infusion bag and tubes, and in particular, on the plastic container and the infusion bag and tubes. This implies that there is a risk of decrease in the effective amount of the soluble thrombomodulin in actual administration. Known anti-adsorption means that have been reported include use of basic amino acid for preventing the adsorption of secretin (Japanese Patent Application Laid-Open No. 57(1982)-169425) and use of a cellulose derivative, a nonionic surface-active agent, or methylcyclodextrin for preventing the adsorption of the secretin, insulin and other low molecular weight peptides (Japanese Patent Application Laid-Open Nos. 58(1983)-206513, 59-76024, and 60(1985)-100524). No technique, however, has so far been disclosed for the prevention of the adsorption of the soluble thrombomodulin.

SUMMARY OF THE INVENTION

An object of the present invention is to provide a highly stable soluble thrombomodulin-containing composition which can be stored for a prolonged period. Another object of the present invention is to provide a highly stable soluble thrombomodulin-containing composition which would not exhibit adsorption of the soluble thrombomodulin onto the surface of the container after diluting to a lower concentration. More illustratively, the object of the present invention is to provide a lyophilized soluble thrombomodulin-containing composition that can be used as a highly safe, stable medicament even after storing for a prolonged period at room temperature. Furthermore, the object of the present invention is to provide a lyophilized soluble thrombomodulin-containing composition that can be used as a medicament that would not undergo decrease in the amount of the soluble thrombomodulin by the adsorption of the soluble thrombomodulin onto the container after diluting into an aqueous solution of a low concentration.

A further object of the present invention is to provide a stabilizing agent and a method for stabilizing the soluble thrombomodulin.

A still further object of the present invention is to provide an anti-adsorption agent and a method for preventing adsorption of the soluble thrombomodulin.

In order to solve the problems associated with the insufficient stability of the soluble thrombomodulin, the inventors of the present invention have made an intensive study of the soluble thrombomodulin, and in particular, in the lyophilized soluble thrombomodulin composition, and found that admixing of maltose (which may be α- or β-maltose or a mixture thereof at any desired mixing ratio; unless otherwise noted, the term, maltose includes all of these species), lactose (which may be α- or β- lactose or a mixture thereof at any desired mixing ratio; unless otherwise noted, the term, lactose includes all of these species), sucrose, or arginine (which may be D- or L-arginine or a racemic form thereof; unless otherwise noted, the term, arginine includes all of these species), or a salt thereof with the soluble thrombomodulin is highly effective in stabilizing the soluble thrombomodulin, and particularly, in stabilizing the soluble thrombomodulin for a prolonged period of time. It has also been found that a nonionic surface-active agent is effective in preventing the adsorption of the soluble thrombomodulin onto the surface of the container after its dilution to a low concentration. The present invention has been completed on such findings.

In view of the above findings, there is provided in accordance with the present invention a soluble thrombomodulin-containing composition comprising a soluble thrombomodulin and at least one member selected from maltose, lactose, sucrose, and arginine and a salt thereof as critical components; a thrombomodulin-containing composition further comprising a nonionic surface-active agent; and thrombomodulin-containing composition comprising a soluble thrombomodulin and a nonionic surface-active agent as critical components.

The soluble thrombomodulin may preferably be a human urine soluble thrombomodulin. It is also preferable to use a recombinant human soluble thrombomodulin for the soluble thrombomodulin. Accordingly, the soluble thrombomodulins that may be used in the present invention include those described in the known references cited in the foregoing "Background Art", which are incorporated herein by reference.

The human urine soluble thrombomodulin may preferably have a partial structure and properties as described below:

a) molecular weight: 72,000 ± 3,000
[measured by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) under non-reduced condition];
b) isoelectric point: 3.9 ± 0.2;
c) terminal amino acid sequence
N terminal:

Ala-Pro-Ala-Glu-Pro-Gln-Pro-Gly-Gly-
Ser-Gln-Cys-Val-Glu-His-Asp-Cys-Phe-Ala-Leu-Tyr-
Pro-Gly-Pro-Ala-Thr-Phe-Leu-,

and
C terminal: -Leu-Ala-Arg or -Leu-Val-Arg; and
d) sugar composition (% by weight):
neutral sugar: 5.5 ± 1.0%
[measured by phenol sulphuric acid method],
amino sugar 2.2 ± 1.0%
[measured by Elson-Morgan's method (Blix's modification)], and
sialic acid: 2.8 ± 1.5%
[measured by Warren's method].

Alternatively, the human urine soluble thrombomodulin may preferably have a partial structure and properties as described below:

a) molecular weight: 79,000 ± 3,000
[measured by SDS-PAGE under non-reduced condition];
b) isoelectric point: 3.8 ± 0.2;
c) terminal amino acid sequence N terminal:

Ala-Pro-Ala-Glu-Pro-Gln-Pro-Gly-Gly-
Ser-Gln-Cys-Val-Glu-His-Asp-Cys-Phe-Ala-Leu-Tyr-
Pro-Gly-Pro-Ala-Thr-Phe-Leu-,

and
C terminal: -Leu-Ala-Arg or -Leu-Val-Arg; and
d) sugar composition (% by weight):
neutral sugar: 6.2 ± 1.0%
[measured by phenol sulphuric acid method],
amino sugar 3.1 ± 1.0%
[measured by Elson-Morgan's method (Blix's modification)], and
sialic acid: 3.8 ± 1.5%
[measured by Warren's method].

According to the present invention, there is also provided a soluble thrombomodulin-containing composition comprising two or more molecular species of soluble thrombomodulins and at least one member selected from maltose, lactose, sucrose, and arginine and a salt thereof as critical components; a thrombomodulin-containing composition further comprising a nonionic surface-active agent; and a thrombomodulin-containing composition comprising soluble thrombomodulins and a nonionic surface-active agent as critical components. The preferable species of the soluble thrombomodulins used for the composition are the same as those used for the composition of the above-described aspect of the present invention.

The soluble thrombomodulin-containing composition according to the above-described aspects of the present invention may preferably be in the form of a lyophilized composition.

According to the present invention, there is also provided a use of the composition wherein the soluble thrombomodulin-containing composition comprising a soluble thrombomodulins and at least one member selected from maltose, lactose, sucrose, and arginine and a salt thereof as critical components; and a nonionic surface-active agent are separately prepared, and mixed immediately before the administration of the composition. The preferable species of the soluble thrombomodulins used for the composition are the same as those used for the composition of the above-described aspect of the present invention.

According to the present invention, there is also provided a method for producing a soluble thrombomodulin-containing composition comprising the step of preparing a solution of a soluble thrombomodulin and at least one member selected from the group consisting of maltose, lactose, sucrose, arginine and a salt thereof, and a nonionic surface-active agent.

According to the present invention, there is also provided a method for producing a soluble thrombomodulin-containing composition comprising the steps of preparing a solution of a soluble thrombomodulin and at least one member selected from the group consisting of maltose, lactose, sucrose, arginine and a salt thereof, and a nonionic surface-active agent; and lyophilizing the soluble thrombomodulin-containing composition in the form of the solution.

According to the present invention, there is also provided a method for producing a soluble thrombomodulin-containing composition comprising the step of preparing a solution of two or more molecular species of soluble thrombomodulins and at least one member selected from the group consisting of maltose, lactose, sucrose, arginine and a salt thereof, and a nonionic surface-active agent.

According to the present invention, there is also provided a method for producing a soluble thrombomodulin-containing composition comprising the steps of preparing a solution of two or more molecular species of soluble thrombomodulins and at least one member selected from the group consisting of maltose, lactose, sucrose, arginine and a salt thereof, and a nonionic surface-active agent; and lyophilizing the soluble thrombomodulin-containing composition in the form of the solution.

According to the present invention, there is also provided a method for stabilizing a soluble thrombomodulin comprising the step of adding at least one member selected from maltose, lactose, sucrose, and arginine and a salt thereof to the soluble thrombomodulin. According to the present invention, there is also provided a stabilizing agent for a soluble thrombomodulin comprising at least one member selected from maltose, lactose, sucrose, and arginine and a salt thereof. According to the present invention, there is also provided a method for preventing adsorption of a soluble thrombomodulin comprising the step of adding a nonionic surface-active agent to the soluble thrombomodulin; and an anti-adsorption agent for a soluble thrombomodulin comprising a nonionic surface-active agent.

According to the present invention, there is also provided a method for stabilizing two or more molecular species of soluble thrombomodulins comprising the step of adding at least one member selected from maltose, lactose, sucrose, and arginine and a salt thereof to the two or more molecular species of soluble thrombomodulins; and a method for preventing adsorption of two or more molecular species of soluble thrombomodulins comprising the step of adding a nonionic surface-active agent to the two or more molecular species of soluble thrombomodulins.

According to the present invention, there is also provided a soluble thrombomodulin-containing pharmaceutical composition comprising a pharmaceutically effective amount of a soluble thrombomodulin and at least one member selected from maltose, lactose, sucrose, and arginine and a salt thereof as critical components; a soluble thrombomodulin-containing pharmaceutical composition as described above further comprising a pharmaceutically acceptable nonionic surfactant; and a soluble thrombomodulin-containing pharmaceutical composition comprising a pharmaceutically effective amount of a soluble thrombomodulin and a pharmaceutically acceptable nonionic surface-active agent. According to the present invention, there is also provided a prophylactic/therapeutic agent for blood coagulation disorder-related diseases that is highly stable even after storing for a prolonged period of time and wherein the soluble thrombomodulin is prevented from being adsorbed onto the surface of the container after its dilution to a low concentration. The preferable species of the soluble thrombomodulins used are the same as those used for the composition of the above-described aspect of the present invention.

BEST MODE FOR CARRYING OUT THE INVENTION

The present invention is hereinafter described in further detail.

The soluble thrombomodulin used in the present invention may be either a natural soluble thrombomodulin or a genetically engineered soluble thrombomodulin. The genetically engineered soluble thrombomodulin may also be a modified soluble thrombomodulin or a chimeric soluble thrombomodulin. Typical soluble thrombomodulins are those described in conjunction with the prior art. When the composition of the present invention is used for a medicament, use of a soluble thrombomodulin of human origin is preferred, and in particular, use of a soluble thrombomodulin of human urine origin is preferred.

Typical natural soluble thrombomodulins include those described in Japanese Patent Application Laid-Open No. 3(1991)-218399 having a molecular weight under non-reduced condition of 72,000 ± 3,000 (hereinafter referred to as UTM1) and 79,000 ± 3,000 (hereinafter referred to as UTM2). Typical genetically engineered soluble thrombomodulins

include recombinant human urine soluble thrombomodulins described in WO92/00325, and soluble thrombomodulin described in Japanese Patent Application Laid-Open No. 1(1989)-6219 comprising 498 amino acid residues with the amino terminal amino acid sequence of Ala-Pro-Ala-. When the soluble thrombomodulin is used for a medicament, the soluble thrombomodulin should be purified to a pharmaceutically acceptable level.

In the composition of the present invention, the soluble thrombomodulin may be used either alone or in a combination of two or more molecular species of the soluble thrombomodulins mixed at any desired mixing ratio. Typical such combinations are combination of 2 types of human urine soluble thrombomodulins disclosed in Japanese Patent Application Laid-Open No. 3(1991)-218399, and combination of soluble thrombomodulins having different sugar chain structures as disclosed in WO91/04276.

The natural soluble thrombomodulin may be produced in accordance with the method described in Japanese Patent Application Laid-Open No. 3(1991)-218399 or 3(1991)-86900. The recombinant soluble thrombomodulin may be produced in accordance with the method described WO92/00325, Japanese Patent Application Laid-Open No. 1(1989)-6219, or WO91/04276.

The stabilizing agent used in the present invention may be a disaccharide having reducibility, sucrose, or an amino acid. More illustratively, in the present invention, at least one member selected from maltose, lactose, sucrose, and arginine and a salt thereof is used as the stabilizing agent in stabilizing the soluble thrombomodulin. The salt of the arginine may be either a salt with an inorganic acid or with an organic acid so long as the salt is pharmaceutically acceptable. Exemplary preferable salts are hydrochloride, citrate, and sulfate, the hydrochloride being the most preferred. The amount of such stabilizing agent used is not limited to any particular range. Typical amount used is in the range of from about 0.1 mg to about 1,000 mg per 1 mg titer of the soluble thrombomodulin. The amount used may preferably be from about 0.5 mg to about 500 mg, and more preferably, from about 0.5 mg to about 100 mg per 1 mg titer of the soluble thrombomodulin. When the composition is to be lyophilized and the additive used for the stabilization of the soluble thrombomodulin is sucrose, use of a somewhat smaller amount is preferred since a lyophilized composition containing the sucrose at a high mixing ratio in relation to the soluble thrombomodulin may collapse during its storage. Preferred amount of the sucrose used is in the range of from about 0.5 mg to about 50 mg per 1 mg titer of the soluble thrombomodulin. If desired, a high molecular weight compound such as dextran may be used in combination with the sucrose in order to prevent such collapse.

It should be noted that the stabilizing agent selected from maltose, lactose, sucrose, and arginine and a salt thereof is preferably used at an amount of 100 mg or less per 1 ml of the solution (composition) containing the soluble thrombomodulin. In addition to the effect of stabilizing the soluble thrombomodulin, such stabilizing agent may additionally serve as a diluent, buffering agent, isotonizing agent, dispersing agent, or the like depending on the amount used. Therefore, the amount of such additive used should be determined by taking the intended use of the resulting composition into consideration.

The anti-adsorption agent used in the present invention is a surface-active agent which may preferably be a nonionic surface-active agent. The nonionic surface-active agent may preferably the one that is pharmaceutically acceptable, and it not limited to any particular species. Exemplary preferable surface-active agent include an ethylene oxide-propylene oxide copolymer, a poly(oxyalkylene) mono- or tri-sorbitan ester (a fatty acid ester of sorbitol and an anhydride thereof that has been copolymerized with various molar numbers of ethylene oxide), polyoxyethylene-hardened caster oil, and the like. Typical ethylene oxide-propylene oxide copolymers include Pluronic F68, Poloxamer 188, etc.; and typical poly(oxyalkylene) mono- or tri-sorbitan esters include Polysorbate 80 (olate ester), Polysorbate 20 (laurate ester), Polysorbate 40 (palmitate ester), Polysorbate 69 (stearate ester), etc.; and typical polyoxyethylene-hardened caster oils include HCO40, HCO60, etc. One or more nonionic surface-active agents selected from the above-mentioned species may be used in the present invention, and when one nonionic surface-active agent is used, it is preferable to use an ethylene oxide-propylene oxide copolymer or a poly(oxyalkylene) mono- or tri-sorbitan ester; and more preferably, Pluronic F68, Polysorbate 80, or Polysorbate 20; and most preferably, Pluronic F68.

The amount of the nonionic surface-active agent added as an anti-adsortpion agent is not limited to any particular range. When the soluble thrombomodulin-containing composition is in the form of an aqueous solution, the nonionic surface-active agent may be desirably used at a concentration of 0.00005% by weight or higher. In other words, the nonionic surface-active agent may be desirably used at a sufficiently low concentration at which the nonionic surface-active agent itself would not exhibit any pharmaceutical activity in the living body after its administration. In conjunction with such consideration, the nonionic surface-active agent may be desirably used in an aqueous solution of the soluble thrombomodulin of at a concentration of 1% by weight or less. The effectivity of the anti-adsorption component may vary in accordance with its concentration and with the material and surface area of the container, and therefore, the nonionic surface-active agent may be used at a suitable amount in accordance with the dilution ratio of the composition in clinical use, or material and size of the container used for the dilution. Preferably, use of the nonionic surface-active agent at an amount that would result in the above-described concentration of from 0.00005 to 1% by weight is suitable for attaining the objects of the present invention. In addition, it is preferable to use the nonionic surface-active agent at an amount that would reach a concentration of from 0.0001 to 0.01% by weight in the body when its administration.

The maltose, lactose, sucrose, and arginine and a salt thereof, or a nonionic surface-active agent used in the present invention as an essential component for stabilization or anti-adsorption may be used either alone or as a combination of two or more. Typical combinations include maltose and a nonionic surface-active agent; lactose and a nonionic surface-active agent; sucrose and a nonionic surface-active agent; arginine and a nonionic surface-active agent; maltose and arginine; lactose and arginine; sucrose and arginine; maltose and lactose; maltose and sucrose; lactose and sucrose; maltose, lactose, and sucrose; maltose, arginine and a nonionic surface-active agent; lactose, arginine and a nonionic surface-active agent; and sucrose, arginine and a nonionic surface-active agent; and mixing ratio of the components is not limited to any particular range. In the above-mentioned combinations, the nonionic surface-active agent may be used either alone or in combination of two or more.

Of the above-mentioned combinations, the preferred are combinations of at least one member selected from maltose, lactose, sucrose and arginine together with a nonionic surface-active agent, and preferred nonionic surface-active agents used in such combinations are Pluronic F68, Polysorbate 80 and Polysorbate 20, among which Pluronic F68 being the most preferred. In other words, the most preferable combinations are maltose and Pluronic F68; lactose and Pluronic F68; sucrose and Pluronic F68; and arginine and Pluronic F68, which may be mixed at any desired mixing ratio.

The soluble thrombomodulin-containing composition of the present invention may contain any desired stabilizers, preservatives, antiseptics, buffering agents, thickening agents, surface-active agents, or the like that is required for the intended use of the composition in addition to the soluble thrombomodulin and the at least one critical component selected from maltose, lactose, sucrose, and arginine and a salt thereof. Alternatively, the soluble thrombomodulin-containing composition of the present invention may contain any desired stabilizers, preservatives, antiseptics, buffering agents, thickening agents, surface-active agents, or the like that is required for the intended use of the composition in addition to the soluble thrombomodulin, the at least one critical component selected from maltose, lactose, sucrose, and arginine and a salt thereof, and the nonionic surface-active agent. The soluble thrombomodulin-containing composition of the present invention may contain any desired stabilizers, preservatives, antiseptics, buffering agents, thickening agents, surface-active agents, or the like that is required for the intended use of the composition in addition to the soluble thrombomodulin and the nonionic surface-active agent. The lyophilized preparation used for medication may contain any of preservatives, stabilizers, binders, diluents, disintegrants, moistening agents, lubricants, coloring agents, aromatic agents, flavoring agents, suspending agents, emulsifiers, solubilizers, buffering agents, isotonizing agents, surface-active agents, adsorption-preventing agents, soothing agents, and the like in accordance with the intended use of the preparation. In particular, inclusion in the preparation of a buffering agent for pH adjustment and a isotonizing agent for osmotic pressure adjustment is preferred. Although the type and the amount of the above-mentioned additives do not essentially influence the nature of the present invention, use of salf of such additive at an excessively high concentration is not preferable in view of the inhibition of the cake formation during lyophilization.

The soluble thrombomodulin-containing composition of the present invention may be produced by dissolving at least one member selected from maltose, lactose, sucrose, arginine and a salt thereof, and a nonionic surface-active agent in a solution containing the soluble thrombomodulin that has been prepared as described above to thereby produce a solution.

Alternatively, the soluble thrombomodulin-containing composition of the present invention may be produced by mixing the lyophilized soluble thrombomodulin with the at least one member selected from maltose, lactose, sucrose, arginine and a salt thereof, and a nonionic surface-active agent; and dissolving the mixture in a suitable solvent such as distilled water or physiological saline, or alternatively, in a suitable buffer solution. In either case, the resulting soluble thrombomodulin-containing composition comprises the soluble thrombomodulin and the at least one member selected from maltose, lactose, sucrose, arginine a salt thereof, and a nonionic surface-active agent dissolved in a solution. It should also be noted that, in either case, the at least one member selected from maltose, lactose, sucrose, arginine and a salt thereof, and a nonionic surface-active agent may be dissolved in a suitable solvent before mixing with the soluble thrombomodulin. When the soluble thrombomodulin-containing composition is used as a medicament, the additives used should be of pharmaceutically acceptable grade.

The thus prepared solution may be lyophilized by a conventional procedure to prepare the composition in cake or powder form. When the soluble thrombomodulin-containing composition is used as a medicament, the solution may preferably be aseptically filtered and filled in vials, ampoules, or the like, and if desired, the thus filled solution may be lyophilized by a conventional procedure to produce a lyophilized preparation.

When the soluble thrombomodulin-containing composition is used as a medicament, it may be administered by a conventional administration procedure, namely, parenteral administration such as intravenous, intramuscular, or subcutaneous administration. The thrombomodulin-containing composition in the form of lyophilized preparation may be dissolved in water for injection before its use, and then administered to the patient. Oral administration is not effective since the administered medicament would be subject to decomposition in digestive tract. However, the composition can be orally administered if the composition is incorporated in liposomes, microspheres, nanospheres or the like that are less likely to be decomposed in the digestive tract. The composition can also be administered permucosally from mucous membrane in rectum, nasal cavity, or hypoglottis.

Typical daily dose is 0.005 to 500 mg titer, and preferable daily dose is 0.1 to 10 mg titer as disclosed in Japanese Patent Application Laid-Open No. 3(1991)-218399. The dose, however, may be suitably adjusted by taking the age, weight, conditions and the like of the patient into consideration.

The thus produced composition of the present invention is stable throughout the processes of freezing, drying, storage, temperature elevation, and dissolution, and has an excellent long term storability at room temperature. The stabilizing agent and the anti-adsorption agent that have been found effective for use in the soluble thrombomodulin composition of the present invention are highly safe, and when the composition of the present invention is used for a medicament, such additives enable the high quality of the medicament to be retained for a prolonged period with no risk of inactivation or aggregate formation. In addition, when the composition of the present invention is diluted to constitute an aqueous solution of low concentration, the soluble thrombomodulin is prevented from being adsorbed onto the container surface, and therefore, the composition of the present invention would not undergo a decrease in the effective amount of the soluble thrombomodulin even when it is diluted with an infusion at clinical sites. Therefore, the soluble thrombomodulin-containing composition of the present invention would constitute a highly safe, room temperature-storable prophylactic or therapeutic agent that can be used for blood coagulation disorder-related diseases. The soluble thrombomodulin-containing composition of the present invention would also constitute a prophylactic or therapeutic agent that can be used for blood coagulation disorder-related diseases wherein the adsorption of the effective component onto the container surface is prevented. The composition, the production method, the stabilization agent, the stabilization method, the anti-adsorption agent, and the anti-adsorption method of the present invention may also be utilized in the purification of the soluble thrombomodulin, or in the storage of soluble thrombomodulin stock.

EXAMPLES

The present invention is hereinafter described in further detail by referring to the Examples.

(Preparation of soluble thrombomodulin-1)
Purification of human urine soluble thrombomodulin

Human urine soluble thrombomodulin was prepared in accordance with the procedure described in Japanese Patent Application Laid-Open No. 3(1991)-218399. 100 liters of stock urine was adjusted to pH 8.5 with 10% NaOH and the precipitate was removed. The urine was then adjusted to pH 5.5 with 4M HCl, and filtered through acrylonitrile fiber to adsorb and remove urokinase in the urine. The filtrate urine was desalted and concentrated by ultrafiltration through a ultrafiltration membrane of 40,000-molecular weight cutoff.

The urine concentrate was adjusted to pH 7.3, and heated to 60°C for 15 minutes. The urine was then passed through a 300 ml column of DEAE-cellulose (manufactured by Whatman) that had been conditioned with 0.05M phosphate buffered saline, pH 6.5 containing 0.068M NaCl for adsorption of the active fraction in the urine. The column was washed with 750 ml of the buffer which was the same as the one used for the conditioning of the column, and subsequently, the adsorbed active fraction was eluted from the column with acetate buffer, pH 4.0 containing 0.05M NaCl.

The eluate was concentrated with a ultrafiltration membrane of 30,000-molecular weight cutoff, and adjusted to pH 7.5 with 2M NaOH, and passed through a 2.5 ml column of DIP-thrombin-agarose that had been conditioned with 0.02M Tris-HCl buffer, pH 7.5 containing 0.1M NaCl, 1mM benzamidine hydrochloride, and 0.5mM $CaCl_2$ for adsorption of the active fraction.

The column was then washed with 25 ml of the buffer which was the same as the one used for the conditioning of the column, and the adsorbed active fraction was eluted from the column with 0.02M Tris-HCl buffer, pH 7.5 containing 1M NaCl, 1mM benzamidine hydrochloride, and 0.5mM EDTA. The eluate was then dialyzed against the buffer which was the same as the one used for the conditioning of the column, and purified by DIP-thrombin-agarose chromatography on a column which had a volume the same as the one used in the previous DIP-thrombin-agarose chromatography and which had been similarly conditioned. The same column was used for the second DIP-thrombin-agarose chromatography, and the column was washed with 10 ml of the buffer which was the same as the one used for the conditioning of the column, and then, with 10 ml of 0.02M Tris-HCl buffer, pH 7.5 containing 0.8M NaCl, 1mM benzamidine hydrochloride, and 0.5mM $CaCl_2$. The adsorbed active fraction was eluted from the column with 0.02M Tris-HCl buffer, pH 7.5 containing 1M NaCl, 1mM benzamidine hydrochloride, and 0.5mM EDTA.

The eluate was concentrated with a ultrafiltration membrane of 30,000-molecular weight cutoff, and the concentrate was subjected to gel filtration on 500 ml column of Sephacryl S-300 (manufactured by Pharmacia Fine Chemicals) that had been conditioned with 0.01M phosphate buffered saline, pH 7.0 containing 0.14M NaCl to collect the active fraction (UTM0). In other series of purification, an active fraction (UTM1) corresponding to a molecular weight of 72,000 ± 3,000, and an active fraction (UTM2) corresponding to a molecular weight of 79,000 ± 3,000 as measured by SDS-PAGE under non-reduced condition were collected. The thus obtained fractions were dialyzed against distilled water overnight, and lyophilized.

The resulting natural human urine soluble thrombomodulins, i.e. UTM1 and UTM2 had the partial structure and the properties as described below.

(1) UTM1

a) molecular weight: 72,000 ± 3,000
[measured by sodium dodecyl sulfate (SDS) - polyacrylamide gel electrophoresis (PAGE) under non-reduced condition];
b) isoelectric point: 3.9 ± 0.2;
c) terminal amino acid sequence (amino acid sequence of SEQ ID NO. 1 in th Sequence Listing)
N terminal:

Ala-Pro-Ala-Glu-Pro-Gln-Pro-Gly-Gly-
Ser-Gln-Cys-Val-Glu-His-Asp-Cys-Phe-Ala-Leu-Tyr-
Pro-Gly-Pro-Ala-Thr-Phe-Leu-,

and
C terminal: -Leu-Ala-Arg or -Leu-Val-Arg; and
d) sugar composition (% by weight):
neutral sugar: 5.5 ± 1.0%
[measured by phenol sulphuric acid method],
amino sugar 2.2 ± 1.0%
[measured by Elson-Morgan's method (Blix's modification)], and
sialic acid: 2.8 ± 1.5%
[measured by Warren's method].

(2) UTM2

a) molecular weight: 79,000 ± 3,000
[measured by SDS-PAGE under non-reduced condition];
b) isoelectric point: 3.8 ± 0.2;
c) terminal amino acid sequence (amino acid sequence of SEQ ID NO. 1 in th Sequence Listing)
N terminal:

Ala-Pro-Ala-Glu-Pro-Gln-Pro-Gly-Gly-
Ser-Gln-Cys-Val-Glu-His-Asp-Cys-Phe-Ala-Leu-Tyr-
Pro-Gly-Pro-Ala-Thr-Phe-Leu-,

and
C terminal: -Leu-Ala-Arg or -Leu-Val-Arg; and
d) sugar composition (% by weight):
neutral sugar: 6.2 ± 1.0%
[measured by phenol sulphuric acid method],
amino sugar 3.1 ± 1.0%
[measured by Elson-Morgan's method (Blix's modification)], and
sialic acid: 3.8 ± 1.5%
[measured by Warren's method].

(Preparation of soluble thrombomodulin-2)
Preparation of genetically engineered recombinant human soluble thrombomodulin (RTM1)

Recombinant human soluble thrombomodulin was prepared in accordance with the method described in WO92/00325. A vector expressing soluble thrombomodulin (ruTM-Ala) comprising 456 amino acid residues was prepared by utilizing a DNA probed from human placental cDNA library, and the resulting vector was introduced in CHO cell. Gene amplification was conducted to produce a cell line of high expression. The culture medium of the high expression cell line was purified by DIP-thrombin-agarose column chromatography and gel filtration to obtain the desired product (RTM1).

(Preparation of soluble thrombomodulin-3)
Preparation of genetically engineered recombinant human soluble thrombomodulin (RTM2)

Recombinant human soluble thrombomodulin was prepared in accordance with the method described in WO92/00325. A vector expressing soluble thrombomodulin comprising 498 amino acid residues having amino terminal amino acid sequence of Ala-Pro-Ala- was prepared by utilizing a DNA probed from human placental cDNA library, and the resulting vector was introduced in CHO cell. Gene amplification was conducted to produce a cell line of high expression. The culture medium of the high expression cell line was purified by DIP-thrombin-agarose column chromatography and gel filtration to obtain the desired product (RTM2).

The present invention is further described by referring to the following experiments, which by no means limit the scope of the invention.

(Experiment 1)

UTM0 prepared in the above-described "Preparation of soluble thrombomodulin-1" was used to prepare the lyophilized injections as shown below. The injections were stored in an incubator at 50°C, and evaluated for their residual titer after storing for 3 and 6 months, and for their aggregation formation rate after storing for 6 months by the procedure as described below. The results are shown in Tables 1 and 2. It should be noted that the percentage of the residual titer shown in Table 1 is the percentage of the residual titer after storing at 50°C in relation to the residual titer of the same injection after storing at 4°C for the same period. The UTM0 used in the experiment contained 69% of the UTM1 and 31% of the UTM2.

Preparation 1

In 30 ml of distilled water adapted for use in preparing injections were dissolved 75 mg titer of UTM0 and 300 mg of maltose. The resulting solution was aseptically filtered, and the filtrate was filled in sterilized glass vials in 1 ml portions. The content of the vials was then lyophilized to prepare the injection that is to be dissolved before its use.

The above-described procedure was repeated by using the ingredients as described below to prepare Preparations 2 to 9.

Preparation 2

UTM0	75 mg titer
Lactose	300 mg

Preparation 3

UTM0	75 mg titer
Sucrose	300 mg

Preparation 4

UTM0	75 mg titer
Arginine hydrochloride	600 mg

Preparation 5

UTM0	75 mg titer
Glucose	300 mg

Preparation 6

UTM0	75 mg titer
Mannitol	300 mg

Preparation 7

UTM0	75 mg titer
Glycine	600 mg

Preparation 8

UTM0	75 mg titer
Purified gelatin	600 mg

Preparation 9

UTM0	75 mg titer
Human serum albumin	300 mg

(Measurement of titer)

The preparations were evaluated for their ability to activate protein C in the presence of thrombin by using Glu-Pro-Arg-p-NA (manufactured by Kabi) for the synthetic substrate. Human urine soluble thrombomodulin (UTM0) purified by Mochida Pharmaceutical Co., Ltd. was used for the standard.

The standard soluble thrombomodulin and the soluble thrombomodulin-containing preparations were respectively diluted with 0.05% Tween 20/Tris-HCl buffer, pH 8.4 to a suitable concentration. To 20 µl of the dilution was added 60 µl of 20mM $CaCl_2$/Tris-HCl buffer, pH 8.4, and then 20 µl of bovine thrombin (manufactured by Mochida Pharmaceutical Co., Ltd.), and the reaction was promoted at room temperature for 20 minutes. To the reaction mixture was added 20 µl of 12 U/ml solution of human protein C (manufactured by American Diagnostica), and the reaction was promoted at room temperature for 20 minutes. To the reaction solution was added 80 µl of mixed solution of human antithrombin III (manufactured by The Green Cross Corporation) and heparin (manufactured by Mochida Pharmaceutical Co., Ltd.) to a final concentrations of 0.15 U/ml and 15 U/ml, respectively, and the reaction was promoted at room temperature for 20 minutes. 125 µl of the reaction solution was aliquoted, and 125 µl of 3mM solution of the synthetic substrate was added to the aliquoted reaction solution. Absorption at a wave length of 405 nm was continuously measured at room temperature to determine initial reaction rate. A calibration curve was depicted by using the standard solution, and the titer of the preparations was evaluated by referring to the calibration curve. The thus determined titer was converted to the titer of rabbit lung thrombomodulin according to the description of Japanese Patent Application Laid-Open No. 3(1991)-218399.

(Measurement of aggregation formation rate)

Aggregation formation rate of the soluble thrombomodulin was measured by gel filtration using TSK-gel™ G3000 SW_{XL} (manufactured by Toyo Soda Mfg. Co., Ltd.).

Table 1

	Additive		Residual titer (%)	
	Type	Amt. (mg)	3 months	6 months
Examples				
Preparation 1	maltose	300	99.6	98.9
Preparation 2	lactose	300	99.8	99.3
Preparation 3	sucrose	300	98.4	98.7
Preparation 4	arginine hydrochloride	600	99.6	99.6
Comparative Examples				

Table 1

Preparation 5	glucose	300	96.0	97.6
Preparation 6	mannitol	300	91.3	89.3
Preparation 7	glycine	600	92.9	82.2
Preparation 8	purified gelatin	600	89.0	95.6
Preparation 9	HSA	300	87.7	84.8

Table 2

	Additive		Aggregation formation rate (%)	
	Type	Amt. (mg)	Immediately after lyophilization	After 6 months
Examples				
Preparation 1	maltose	300	0.0	0.8
Preparation 2	lactose	300	-	0.0
Preparation 3	sucrose	300	-	0.6
Preparation 4	arginine hydrochloride	600	0.0	0.0
Comparative Examples				
Preparation 5	glucose	300	1.4	3.4
Preparation 6	mannitol	300	0.0	4.0
Preparation 7	glycine	600	-	5.7

As shown in Tables 1 and 2, the stabilization effect of the additive on the human urine soluble thrombomodulin was significant in the case of maltose, lactose, sucrose, and arginine hydrochloride compared to the case of other common additives such as glucose, mannitol, glycine, purified gelatin, and human serum albumin (HSA). In other words, the human soluble thrombomodulin underwent a significant increase in its storage stability. The stabilization effect was most significant when lactose and arginine hydrochloride were added.

(Experiment 2)

UTM1 and UTM2 prepared in the above-described "Preparation of soluble thrombomodulin-1" were used to prepare the lyophilized compositions as shown below. The compositions were stored in an incubator at a temperature of 40°C and at a humidity of 75%. After storing for 6 months, the compositions were evaluated for their residual titer and aggregation formation rate by repeating the procedure of Experiment 1. The results are shown in Tables 3 and 4. The percentage of the residual titer shown in Table 3 is the percentage of the titer after the storage in relation to the titer before the storage.

Composition 1

In 1 ml of purified water were dissolved 2.5 mg titer of UTM2 and 10 mg of maltose, and the resulting solution was lyophilized.

The above-described procedure was repeated to prepare the Compositions 2 to 8 by using the ingredients as described below.

Composition 2

UTM1	2.5 mg titer
Lactose	10 mg

Composition 3

UTM2	2.5 mg titer
Arginine hydrochloride	20 mg

Composition 4

UTM1	2.5 mg titer
Sucrose	10 mg

Composition 5

UTM1	2.5 mg titer
Mannitol	10 mg

Composition 6

UTM1	2.5 mg titer
Glycine	20 mg

Composition 7

UTM1	2.5 mg titer
Purified gelatin	20 mg

Composition 8

UTM1	2.5 mg titer
HSA	10 mg

Table 3

	Soluble thrombomodulin		Additive		Residual titer (%)	
	Type	mg titer	Type	Amt. (mg)	Immediately after lyophilization	After 6 months
Examples						
Composition 1	UTM2	2.5	maltose	10	100.2	99.1
Composition 2	UTM1	2.5	lactose	10	-	102.3
Composition 3	UTM2	2.5	arginine hydrochloride	20	-	100.9
Composition 4	UTM1	2.5	sucrose	10	-	99.6
Comparative Examples						
Composition 5	UTM1	2.5	mannitol	10	99.8	96.5
Composition 6	UTM1	2.5	glycine	20	-	96.2
Composition 7	UTM1	2.5	purified gelatin	20	102.3	87.3
Composition 8	UTM1	2.5	HSA	10	-	90.9

Table 4

	Soluble thrombomodulin		Additive		Aggregation formation rate (%)
	Type	mg titer	Type	Amt. (mg)	
Examples					
Composition 1	UTM2	2.5	maltose	10	0.7
Composition 2	UTM1	2.5	lactose	10	0.4
Composition 3	UTM2	2.5	arginine hydrochloride	20	0.0
Composition 4	UTM1	2.5	sucrose	10	0.0
Comparative Examples					
Composition 5	UTM1	2.5	mannitol	10	4.0
Composition 6	UTM1	2.5	glycine	20	2.9

As shown in Tables 3 and 4, the stabilization effect of the additive on the human urine soluble thrombomodulin was significant in the case of maltose, lactose, sugar, and arginine hydrochloride compared to the case of other common additives, and the stabilization effect in the case of long term storage was particularly significant.

(Experiment 3)

Evaluation of the stability of the solution

UTM0 prepared in the above-described "Preparation of soluble thrombomodulin-1" was used to prepare 0.05 mg titer/ml soluble thrombomodulin solution containing 0.5 to 5 mg/ml maltose, lactose, sucrose or arginine hydrochloride. The resulting solution was stored at room temperature for 24 hours, and the residual titer was measured after the storage by the procedure described in Experiment 1. No solution exhibited significant loss of the activity.

If the soluble thrombomodulin became denatured to form aggregates, and the composition including such aggregates were introduced into human blood, there is a risk that the aggregates comprising a denatured protein may induce immunological response such as hypersensitivity or thrombosis. Accordingly, low aggregation formation rate is a merit of great importance for a medical preparation used for injection. In developing preparations, storability at room temperature of the preparation is generally determined by evaluating the stability of the preparation after storing 6 month at 40°C. The soluble thrombomodulin-containing preparation of the present invention was quite stable after storing for 6 month under more severe conditions of 50°C as demonstrated in the experiments as described above. The soluble thrombomodulin-containing composition in the form of a solution also had a good storability. Therefore, when the soluble thrombomodulin-containing composition in the form a lyophilized preparation is dissolved before use, the solution can be safely used.

(Experiment 4)

UTM0 prepared in the above-described "Preparation of soluble thrombomodulin-1" was used to prepare the compositions in the form of solution as described below to evaluate the titer. In addition to such solutions, a contrast solution was prepared by dissolving 2.5 mg titer of UTM0 by 2.0 ml of physiological saline, and the contrast solution was also evaluated for its titer. To physiological saline (100 ml) in a plastic container was added 0.24 ml of the thus prepared solution using a syringe to dilute the soluble thrombomodulin to a theoretical final concentration of about 0.003 mg titer/ml. The thus diluted solution was collected 3 hours after the dilution to evaluate the residual titer. The titer was evaluated by repeating the procedure of Experiment 1, above.

Composition 9

2.5 mg titer of UTM0 was mixed with 5 mg of Polysorbate 80, and the mixture was dissolved in 2 ml of physiological saline.

Composition 10

2.5 mg titer of UTM0 was mixed with 10 mg of purified gelatin, and the mixture was dissolved in 2 ml of physiological saline.

Table 5

	Additive		Residual titer (%) after storage in a container (Plastic bottle)
	Type	Final conc. (%)	
Example			
Composition 9	Polysorbate 80	0.0006	96.9
Comparative Example			
Composition 10	purified gelatin	0.0012	87.5
Contrast			
	-	-	79.6

As shown in Table 5, human urine soluble thrombomodulin exhibited a marked adsorption onto the plastic container. Anti-adsorption effect attained by the addition of Polysorbate 80 was more significant than the effect attained by the addition of the pure gelatin.

(Experiment 5)

UTM0 prepared in the above-described "Preparation of soluble thrombomodulin-1" was used to prepare the compositions in the form of solution as described below to evaluate the titer. In addition to such solutions, a contrast solution was prepared by dissolving 2.5 mg titer of UTM0 by 2.0 ml of physiological saline, and the contrast solution was also evaluated for its titer. An infusion system (TERU fusion® TS-A200CK, manufacture by TERUMO) was mounted to a plastic container filled with physiological saline, and 1 ml of the thus prepared solution was added to the physiological saline (500 ml, OTSUKA SEISHOKU CHU manufactured by Otsuka Pharmaceuticals Co., Ltd. in the plastic container) to dilute the soluble thrombomodulin to a theoretical final concentration of about 0.0025 mg titer/ml. The solution that had passed through the infusion set immediately after the dilution and the solution that was directly collected from the plastic bottle were evaluated for their residual titer. The titer was evaluated by repeating the procedure of Experiment 1, above.

Composition 11

2.5 mg titer of UTM0 was mixed with 1 mg of Polysorbate 80, and the mixture was dissolved in 2 ml of physiological saline.

The above-described procedure was repeated to prepare the Compositions 2 to 6 by using the ingredients as described below.

Composition 12

UTM0	2.5 mg titer
Polysorbate 80	0.5 mg

Composition 13

UTM0	2.5 mg titer
Polysorbate 20	1 mg

Composition 14

UTM0	2.5 mg titer
Polysorbate 20	0.5 mg

Composition 15

UTM0	2.5 mg titer
Pluronic F68	5 mg

Composition 16

UTM0	2.5 mg titer
Pluronic F68	1 mg

Table 6

Example	Additive Type	Additive Final conc. (%)	Residual titer (%) after passing through the infusion system	Residual titer (%) after storage in a container (Plastic bottle)
Composition 11	Polysorbate 80	0.0001	87.4	103.3
Composition 12	Polysorbate 80	0.00005	86.2	87.0
Composition 13	Polysorbate 20	0.0001	84.2	91.3
Composition 14	Polysorbate 20	0.00005	83.6	83.4
Composition 15	Pluronic F68	0.0005	94.8	92.2
Composition 16	Pluronic F68	0.0001	92.7	87.5
Contrast	-	-	63.3	79.1

As shown in Table 6, addition of Polysorbate 80, Polysorbate 20 and Pluronic F68 at a concentration of 0.00005% by weight or higher was effective in retaining the activity of the diluted solution of the human urine soluble thrombomodulin both in the infusion set and in the plastic container.

(Experiment 6)

UTM0 prepared in the above-described "Preparation of soluble thrombomodulin-1" was used to prepare the lyophilized injections as shown below. The injections were stored in an incubator at 50°C, and evaluated for their residual titer after storing for 3 and 6 months. The titer was evaluated by repeating the procedure of Experiment 1, above. The results are shown in Table 7. It should be noted that the residual titer is shown in terms of percentage in relation to the titer immediately after the lyophilization.

Preparation 10

In 60 ml of distilled water adapted for use in preparing injections were dissolved 150 mg titer of UTM0, 1200 mg of arginine hydrochloride, and 60 mg of Pluronic F68. The resulting solution was aseptically filtered, and the filtrate was filled in sterilized glass vials in 2 ml portions. The content of the vials was then lyophilized to prepare the injection that is to be dissolved before its use.

The above-described procedure was repeated by using the ingredients as described below to prepare Preparations 11 to 13.

Preparation 11

UTM0	150 mg titer
Arginine hydrochloride	1200 mg
Polysorbate 80	60 mg
Purified gelatin	300 mg

Preparation 12

UTM0	150 mg titer
Maltose	600 mg
Pluronic F68	60 mg

Preparation 13

UTM0	150 mg titer
Maltose	600 mg
Pluronic F68	60 mg
Purified gelatin	300 mg

Table 7

Examples	Additive		Residual titer (%)	
	Type	Amt. (mg)	3 months	6 months
Preparation 10	arginine hydrochloride	1200	100.4	101.0
	Pluronic F68	60		
Preparation 11	arginine hydrochloride	1200	99.3	100.2
	Polysorbate 80	60		
	purified gelatin	300		
Preparation 12	maltose	600	100.4	100.1
	Pluronic F68	60		
Preparation 13	maltose	600	99.2	99.7
	Pluronic F68	60		
	purified gelatin	300		

As shown in Table 7, addition of arginine or maltose in combination with a nonionic surface-active agent resulted in a significant improvement in long-term storability of the human urine soluble thrombomodulin. The results confirmed the availability of a soluble thrombomodulin-containing composition having an excellent stability to endure long-term storage which would not exhibit adsorption of the soluble thrombomodulin on the container surface after dilution to a low concentration.

(Examples of preparations)

The present invention is further illustrated by referring the following examples, which by no means limit the scope of the invention.

(Example 1)

UTM0	10 mg titer
Lactose	100 mg
Purified gelatin	100 mg

The ingredients were dissolved in distilled water adapted for use in preparing injections to make up a total volume of 10 ml, and the resulting solution was aseptically filtered. The filtrate was filled in sterilized vials in 1.0 ml portions, and the content of the vials was lyophilized to prepare the lyophilized soluble thrombomodulin-containing composition.

Example 2

UTM0	25 mg titer
Lactose	100 mg
Pluronic F68	10 mg
Disodium hydrogenphosphate dodecahydrate	0.77 mg
Sodium dihydrogenphosphate dihydrate	0.18 mg
Sodium chloride	2.73 mg

The ingredients were dissolved in distilled water for injections to make up a total volume of 10 ml, and the resulting solution was aseptically filtered. The filtrate was filled in sterilized vials in 1.0 ml portions, and the content of the vials was lyophilized to prepare the lyophilized soluble thrombomodulin-containing composition.

Example 3

UTM0	25 mg titer
L-arginine hydrochloride	200 mg
Polysorbate 80	10 mg
Disodium hydrogenphosphate dodecahydrate	0.77 mg
Sodium dihydrogenphosphate dihydrate	0.18 mg
Sodium chloride	2.73 mg

The ingredients were dissolved in distilled water for injections to make up a total volume of 10 ml, and the resulting solution was aseptically filtered. The filtrate was filled in sterilized vials in 1.0 ml portions, and the content of the vials was lyophilized to prepare the lyophilized soluble thrombomodulin-containing composition.

Example 4

UTM0	25 mg titer
L-arginine hydrochloride	200 mg
Pluronic F68	10 mg

The ingredients were dissolved in distilled water for injections to make up a total volume of 10 ml, and the resulting solution was aseptically filtered. The filtrate was filled in sterilized vials in 1.0 ml portions, and the content of the vials was lyophilized to prepare the lyophilized soluble thrombomodulin-containing composition.

Example 5

UTM0	50 mg titer
Maltose	100 mg
Purified gelatin	100 mg
Disodium hydrogenphosphate dodecahydrate	23.2 mg
Sodium dihydrogenphosphate dihydrate	5.5 mg
Sodium chloride	81.8 mg

The ingredients were dissolved in distilled water for injections to make up a total volume of 10 ml, and the resulting solution was aseptically filtered. The filtrate was filled in sterilized vials in 1.0 ml portions, and the content of the vials was lyophilized to prepare the lyophilized soluble thrombomodulin-containing composition.

Example 6

A lyophilized soluble thrombomodulin-containing composition was prepared by using the same ingredients as Example 5. In the meanwhile, 0.1% aqueous solution of Polysorbate 80 was aseptically prepared, and the solution was dispensed in ampoules in 1.0 ml portions, and the ampoules were melt-sealed to prepare the ampoules having the solution for dissolution filled therein.

Example 7

UTM0	25 mg titer
Sucrose	100 mg
Purified gelatin	100 mg

The ingredients were dissolved in distilled water for injections to make up a total volume of 10 ml, and the resulting solution was aseptically filtered. The filtrate was filled in sterilized vials in 1.0 ml portions, and the content of the vials was lyophilized to prepare the lyophilized soluble thrombomodulin-containing composition.

Example 8

A lyophilized soluble thrombomodulin-containing composition was prepared by using the same ingredients as Example 7. In the meanwhile, 0.1% aqueous solution of Polysorbate 80 was aseptically prepared, and the solution was dispensed in ampoules in 1.0 ml portions, and the ampoules were melt-sealed to prepare the ampoules having the solution for dissolution filled therein.

Example 9

UTM1	25 mg titer
Lactose	800 mg
Purified gelatin	100 mg
Disodium hydrogenphosphate dodecahydrate	23.2 mg
Sodium dihydrogenphosphate dihydrate	5.5 mg

The ingredients were dissolved in distilled water for injections to make up a total volume of 10 ml, and the resulting solution was aseptically filtered. The filtrate was filled in sterilized vials in 1.0 ml portions, and the content of the vials was lyophilized to prepare the lyophilized soluble thrombomodulin-containing composition.

Example 10

UTM2	50 mg titer
L-arginine hydrochloride	200 mg
Purified gelatin	100 mg

The ingredients were dissolved in distilled water for injections to make up a total volume of 10 ml, and the resulting solution was aseptically filtered. The filtrate was filled in sterilized vials in 1.0 ml portions, and the content of the vials was lyophilized to prepare the lyophilized soluble thrombomodulin-containing composition.

Example 11

UTM1	10 mg titer
Sucrose	100 mg
Polysorbate 80	50 mg
Disodium hydrogenphosphate dodecahydrate	23.2 mg
Sodium dihydrogenphosphate dihydrate	5.5 mg

The ingredients were dissolved in distilled water for injections to make up a total volume of 10 ml, and the resulting solution was aseptically filtered. The filtrate was filled in sterilized vials in 1.0 ml portions, and the content of the vials was lyophilized to prepare the lyophilized soluble thrombomodulin-containing composition.

Example 12

UTM2	50 mg titer
L-arginine hydrochloride	200 mg
Purified gelatin	100 mg
Polysorbate 80	10 mg

The ingredients were dissolved in distilled water for injections to make up a total volume of 10 ml, and the resulting solution was aseptically filtered. The filtrate was filled in sterilized vials in 1.0 ml portions, and the content of the vials was lyophilized to prepare the lyophilized soluble thrombomodulin-containing composition.

Example 13

RTM1	25 mg titer
Lactose	200 mg
Polysorbate 80	10 mg
Disodium hydrogenphosphate dodecahydrate	0.77 mg
Sodium dihydrogenphosphate dihydrate	0.18 mg
Sodium chloride	81.8 mg

The ingredients were dissolved in distilled water for injections to make up a total volume of 10 ml, and the resulting solution was aseptically filtered. The filtrate was filled in sterilized vials in 1.0 ml portions, and the content of the vials was lyophilized to prepare the lyophilized soluble thrombomodulin-containing composition.

Example 14

RTM1	25 mg titer
Lactose	200 mg
Pluronic F68	10 mg
Disodium hydrogenphosphate dodecahydrate	0.77 mg
Sodium dihydrogenphosphate dihydrate	0.18 mg
Sodium chloride	81.8 mg

The ingredients were dissolved in distilled water for injections to make up a total volume of 10 ml, and the resulting solution was aseptically filtered. The filtrate was filled in sterilized vials in 1.0 ml portions, and the content of the vials was lyophilized to prepare the lyophilized soluble thrombomodulin-containing composition.

Example 15

RTM2	25 mg titer
Lactose	100 mg
Purified gelatin	100 mg

The ingredients were dissolved in distilled water for injections to make up a total volume of 10 ml, and the resulting solution was aseptically filtered. The filtrate was filled in sterilized vials in 1.0 ml portions, and the content of the vials was lyophilized to prepare the lyophilized soluble thrombomodulin-containing composition.

Example 16

A lyophilized soluble thrombomodulin-containing composition was prepared by using the same ingredients as Example 15. In the meanwhile, 0.1% aqueous solution of Polysorbate 80 was aseptically prepared, and the solution was dispensed in ampoules in 1.0 ml portions, and the ampoules were melt-sealed to prepare the ampoules having the solution for dissolution filled therein.

Example 17

RTM2	10 mg titer
Maltose	100 mg
Purified gelatin	100 mg
Disodium hydrogenphosphate dodecahydrate	0.77 mg
Sodium dihydrogenphosphate dihydrate	0.18 mg
Sodium chloride	81.8 mg

The ingredients were dissolved in distilled water for injections to make up a total volume of 10 ml, and the resulting solution was aseptically filtered. The filtrate was filled in sterilized vials in 1.0 ml portions, and the content of the vials was lyophilized to prepare the lyophilized soluble thrombomodulin-containing composition.

Example 18

RTM2	10 mg titer
Maltose	100 mg
Purified gelatin	100 mg
Pluronic F68	10 mg
Disodium hydrogenphosphate dodecahydrate	0.77 mg
Sodium dihydrogenphosphate dihydrate	0.18 mg
Sodium chloride	81.8 mg

The ingredients were dissolved in distilled water for injections to make up a total volume of 10 ml, and the resulting solution was aseptically filtered. The filtrate was filled in sterilized vials in 1.0 ml portions, and the content of the vials was lyophilized to prepare the lyophilized soluble thrombomodulin-containing composition.

Example 19

UTM0	25 mg titer
L-arginine hydrochloride	100 mg
Lactose	100 mg
Polysorbate 80	10 mg
Disodium hydrogenphosphate dodecahydrate	0.77 mg
Sodium dihydrogenphosphate dihydrate	0.18 mg
Sodium chloride	2.73 mg

The ingredients were dissolved in distilled water for injections to make up a total volume of 10 ml, and the resulting solution was aseptically filtered. The filtrate was filled in sterilized vials in 1.0 ml portions, and the content of the vials was lyophilized to prepare the lyophilized soluble thrombomodulin-containing composition.

Example 20

UTM0	25 mg titer
L-arginine hydrochloride	100 mg
Maltose	100 mg
Pluronic F68	10 mg

The ingredients were dissolved in distilled water for injections to make up a total volume of 10 ml, and the resulting solution was aseptically filtered. The filtrate was filled in sterilized vials in 1.0 ml portions, and the content of the vials was lyophilized to prepare the lyophilized soluble thrombomodulin-containing composition.

Example 21

UTM1	10 mg titer
Lactose	100 mg
Sucrose	100 mg
Polysorbate 80	50 mg
Disodium hydrogenphosphate dodecahydrate	23.2 mg
Sodium dihydrogenphosphate dihydrate	5.5 mg

The ingredients were dissolved in distilled water for injections to make up a total volume of 10 ml, and the resulting solution was aseptically filtered. The filtrate was filled in sterilized vials in 1.0 ml portions, and the content of the vials was lyophilized to prepare the lyophilized soluble thrombomodulin-containing composition.

SEQUENCE LISTING

Sequence ID No.: 1

Sequence length: 28

Sequence type: amino acid

Molecule type: protein

Source organism: human

Sequence: Ala-Pro-Ala-Glu-Pro-Gln-Pro-Gly-
1 5

Gly-Ser-Gln-Cys-Val-Glu-His-Asp-
10 15

Cys-Phe-Ala-Leu-Tyr-Pro-Gly-Pro-
20

Ala-Thr-Phe-Leu-
25

Industrial Utility

As described above, the soluble thrombomodulin-containing composition of the present invention is stable throughout the processes of freezing, drying, storage, temperature elevation, and dissolution. In particular, the lyophilized soluble thrombomodulin-containing composition of the present invention has an excellent long term storability at room temperature. The critical components for the stabilization or the anti-adsorption that have been found in the present invention are highly safe, and when the composition of the present invention is used for a medicament, such additives enable the high quality of the medicament to be retained for a prolonged period with no risk of inactivation or aggregate formation. In particular, the lyophilized soluble thrombomodulin-containing composition of the present invention is sufficiently stable to endure the storage at 50°C for 6 months. In addition, the soluble thrombomodulin-containing composition of the present invention would not exhibit adsorption of the soluble thrombomodulin even when it is diluted to constitute an aqueous solution of a low concentration, and therefore, it can be diluted with an infusion before its administration at clinical sites without undergoing any decrease in the effective amount of the soluble thrombomodulin. Therefore, the soluble thrombomodulin-containing composition of the present invention would constitute a highly safe, room temperature-storable prophylactic or therapeutic agent that can be used for blood coagulation disorder-related diseases. The soluble thrombomodulin-containing composition of the present invention would also constitute a prophylactic or therapeutic agent for blood coagulation disorder-related diseases which would not exhibit adsorption of the soluble thrombomodulin onto the container surface upon clinical use. The soluble thrombomodulin-containing composition, the production method therefor, the stabilization agent, the stabilization method, the anti-adsorption agent, and the anti-adsorption method of the present invention may also be utilized in the purification of the soluble thrombomodulin, and in the storage of soluble thrombomodulin stock.

Claims

1. A soluble thrombomodulin-containing composition characterized in that said composition comprises
   a soluble thrombomodulin, and
   at least one member selected from the group consisting of maltose, lactose, sucrose, and arginine and a salt thereof.

2. A soluble thrombomodulin-containing composition characterized in that said composition comprises
   a soluble thrombomodulin, and
   a nonionic surface-active agent.

3. A soluble thrombomodulin-containing composition characterized in that said composition comprises
   a soluble thrombomodulin,
   at least one member selected from the group consisting of maltose, lactose, sucrose, and arginine and a salt thereof, and
   a nonionic surface-active agent.

4. The soluble thrombomodulin-containing composition according to any one of claims 1 to 3 wherein said soluble thrombomodulin is a human urine soluble thrombomodulin.

5. The soluble thrombomodulin-containing composition according to claim 4 wherein said human urine soluble thrombomodulin is a substance having a partial structure and properties as described below:

   a) molecular weight: 72,000 ± 3,000
   [measured by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) under non-reduced condition];
   b) isoelectric point: 3.9 ± 0.2;
   c) terminal amino acid sequence (amino acid sequence of SEQ ID NO. 1 in th Sequence Listing)
   N terminal:

   Ala-Pro-Ala-Glu-Pro-Gln-Pro-Gly-Gly-
   Ser-Gln-Cys-Val-Glu-His-Asp-Cys-Phe-Ala-Leu-Tyr-
   Pro-Gly-Pro-Ala-Thr-Phe-Leu-,

   and
   C terminal: -Leu-Ala-Arg or -Leu-Val-Arg; and
   d) sugar composition (% by weight):
   neutral sugar: 5.5 ± 1.0%
   [measured by phenol sulphuric acid method],
   amino sugar 2.2 ± 1.0%
   [measured by Elson-Morgan's method (Blix's modification)], and
   sialic acid: 2.8 ± 1.5%
   [measured by Warren's method].

6. The soluble thrombomodulin-containing composition according to claim 4 wherein said human urine soluble thrombomodulin is a substance having a partial structure and properties as described below:

   a) molecular weight: 79,000 ± 3,000
   [measured by SDS-PAGE under non-reduced condition];
   b) isoelectric point: 3.8 ± 0.2;

c) terminal amino acid sequence (amino acid sequence of SEQ ID NO. 1 in th Sequence Listing)
N terminal:

Ala-Pro-Ala-Glu-Pro-Gln-Pro-Gly-Gly-

Ser-Gln-Cys-Val-Glu-His-Asp-Cys-Phe-Ala-Leu-Tyr-

Pro-Gly-Pro-Ala-Thr-Phe-Leu-,

and
C terminal: -Leu-Ala-Arg or -Leu-Val-Arg; and
d) sugar composition (% by weight):
neutral sugar: 6.2 ± 1.0%
[measured by phenol sulphuric acid method],
amino sugar 3.1 ± 1.0%
[measured by Elson-Morgan's method (Blix's modification)], and
sialic acid: 3.8 ± 1.5%
[measured by Warren's method].

7. The soluble thrombomodulin-containing composition according to any one of claims 1 to 3 wherein said soluble thrombomodulin is a recombinant human soluble thrombomodulin.

8. A soluble thrombomodulin-containing composition characterized in that said composition comprises
two or more molecular species of soluble thrombomodulins, and
at least one member selected from the group consisting of maltose, lactose, sucrose, and arginine and a salt thereof.

9. A soluble thrombomodulin-containing composition characterized in that said composition comprises
two or more molecular species of soluble thrombomodulins, and
a nonionic surface-active agent.

10. A soluble thrombomodulin-containing composition characterized in that said composition comprises
two or more molecular species of soluble thrombomodulins,
at least one member selected from the group consisting of maltose, lactose, sucrose, and arginine and a salt thereof, and
a nonionic surface-active agent.

11. The soluble thrombomodulin-containing composition according to any one of claims 1 to 10 wherein said composition has been lyophilized.

12. A method for producing the soluble thrombomodulin-containing composition according to any one of claims 1 to 7 and 11 comprising the step of preparing a solution of at least one species of soluble thrombomodulin, and at least one member selected from the group consisting of maltose, lactose, sucrose, arginine and a salt thereof; and a nonionic surface-active agent.

13. A method for producing the soluble thrombomodulin-containing composition according to claim 12 comprising the step of lyophilizing the soluble thrombomodulin-containing composition in the form of the solution of at least one species of soluble thrombomodulin, and at least one member selected from the group consisting of maltose, lactose, sucrose, arginine and a salt thereof.

14. A stabilizing agent for a soluble thrombomodulin characterized in that said agent comprises at least one member selected from the group consisting of maltose, lactose, sucrose, and arginine and a salt thereof.

15. A method for stabilizing a soluble thrombomodulin characterized in that said method comprises the step of adding at least one member selected from the group consisting of maltose, lactose, sucrose, and arginine and a salt thereof to the soluble thrombomodulin.

16. An anti-adsorption agent for a soluble thrombomodulin characterized in that said agent comprises a nonionic surface-active agent.

17. A method for preventing adsorption of a soluble thrombomodulin characterized in that said method comprises the step of adding a nonionic agent to the soluble thrombomodulin.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP94/02128

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl^6 A61K38/36, 47/26, 47/18, 47/34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int. Cl^6 A61K38/36, 47/26, 47/18, 47/34

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO, A, 93/15755 (Schering AG), August 19, 1993 (19. 08. 93) & AU, A, 9336593	1-17
A	JP, A, 5-58899 (The Green Cross Corp.), March 9, 1993 (09. 03. 93) (Family: none)	1-17
A	WO, A, 91/4276 (Asahi Chemical Industry Co., Ltd.), April 4, 1991 (04. 04. 91) & EP, A, 445304	1-17
A	JP, A, 3-218399 (Mochida Pharmaceutical Co., Ltd.), September 25, 1991 (25. 09. 91) & EP, A, 376251 & US, A, 5202421	1-17

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
March 22, 1995 (22. 03. 95)	April 11, 1995 (11. 04. 95)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

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Chapter 7

Introduction to Formulation of Protein Pharmaceuticals

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1. INTRODUCTION

As the skills of the molecular biologist expand to produce more recombinant protein drugs and those of the protein biochemist increase to produce purer products, the pharmaceutical scientist is faced with greater and more complex formulation challenges. Furthermore, in addition to formulations for the more traditional sterile injectable products, there is now demand for alternate dosage forms such as nasal, rectal, oral, optic, inhalation, and topical. What makes this particularly challenging is that increased purification of the protein product removes it ever further from the natural environment in which it is the most stable. The purification process strips away the carbohydrates, salts, lipids, and other proteins that keep this drug candidate neatly folded into its more thermodynamically favorable shape. A highly purified protein is rendered more sensitive to processes such as shear, agitation, enzymatic and chemical degradation, and aggregation. By understanding the native state of a protein drug candidate and the mechanism by which these processes destabilize it, the pharmaceutical scientist can more successfully

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Stability of Protein Pharmaceuticals, Part B: In Vivo *Pathways of Degradation and Strategies for Protein Stabilization,* edited by Tim J. Ahern and Mark C. Manning. Plenum Press, New York, 1992.

predict formulation excipients and processes that will result in a stable, potent, elegant pharmaceutical dosage form.

This chapter will examine briefly the nature of proteins and the mechanisms of destabilization and, in more detail, the unique methods needed to evaluate the products, as well as the physical and chemical ways to stabilize the proteins in a drug product.

2. NATURE OF PROTEINS AND MECHANISMS OF DESTABILIZATION

Since much has been published on the nature of proteins and the mechanisms of destabilization, this chapter will provide only a brief overview of these for completeness. A polypeptide is a linear sequence of amino acids joined together by a peptide (amide) bond, and can vary from two amino acids to several hundred. Because the properties of these molecules vary with the number of amino acids, traditionally polypeptides have been considered as two groups: peptides (LT 50 aa), which have little secondary structure, and proteins (MT 50 aa), which have secondary, tertiary, and sometimes quaternary structure. Each of the 20 amino acids has its own functional group side chain, and it is from the sequence of these functional groups and their interaction that secondary and tertiary structures result and are stabilized. Generally, because proteins exist in an aqueous environment, the folding of the molecule results in the hydrophobic groups being buried internally and charged or polar functions are exposed on the surface. This results in an energetically favorable state.

Destabilization of protein and peptide molecules is of two types: chemical, which involves changes in covalent bonds, and physical, which involves changes in the spatial three-dimensional structure (denaturation). The chemical degradation mechanisms have been well reviewed (Wang and Hanson, 1988; Manning *et al.*, 1989; Geigert, 1989) and most commonly include hydrolysis (Hammel and Glasstone, 1954; Schultz *et al.*, 1961; Hill, 1965), oxidation, deamidation (Graf *et al.*, 1971; McKerrow and Robinson, 1971; Robinson *et al.*, 1973; Rytting, 1986), disulfide exchange (Kenney *et al.*, 1986), and racemization (Nishi *et al.*, 1980; Masters and Friedman, 1980; Geiger and Clarke, 1987). The physical or denaturation process has also been extensively reviewed (Tanford, 1968, 1969; Timasheff and Fasman, 1969; Pace, 1975; Lapanje, 1978; Schmid, 1979; Privalov, 1979; Klibanov, 1983; Privalov and Gill, 1988; Mozhaev *et al.*, 1988; Jaenicke and Rudolph, 1989; Pace *et al.*, 1989; Timasheff and Arakawa, 1989). This process of unfolding of the molecule resulting in problems of aggregation, adsorption, and loss of activity is of most concern to the formulation scientist, and so is reviewed briefly below.

It is the tertiary structure of the protein that must be stabilized against the various disruptive forces that occur during processing and handling. Potential denaturing forces include chemical stress from factors used in purification, such as pH, ionic strength, or detergents, or physical stress during filtration and filling where surface adsorption and shear contribute to unwinding of the tertiary structure into a random coil. Solvent protein interaction strongly influences conformation. Water, the normal solvent of proteins, forms a hydration layer around the protein and contributes to the hydrogen bonding. The hydrophobic interaction of the amino acid hydrocarbon side chains buried within the molecule provides additional stabilization energy. Disruption of the structure occurs when the solvent partitions the hydrocarbon side chains between a hydrocarbon phase and aqueous phase.

Mozhaev and Martinek (1984) used enzymes from thermophilic bacteria to study structure stability relationships. These enzymes generally maintain activity at temperatures 20–30 ° higher than comparable enzymes from mesophilic bacteria. Thermodynamically, this corresponds to a stabilizing ΔG or 5–7 kcal $mole^{-1}$ and can be achieved by an additional one or two salt bridges within the molecule, or an additional several hydrogen bonds, or seven to ten additional CH_3 groups within the hydrophobic nucleus. By understanding these interactions, the formulation chemist can choose pharmaceutically acceptable excipients to contribute to the thermodynamic stabilization of the protein.

3. METHODS USED TO EVALUATE PROTEIN FORMULATIONS

All of these possibilities need to be accounted for in a well-designed stability evaluation of the protein formulation. A significant difference between protein drugs and the smaller chemical drug molecules is the need for a number of different methodologies to fully evaluate potential degradation pathways (Geigert *et al.*, 1987, 1988). The success of a particular formulation is gauged by its ability to prevent loss of the physical and chemical stability of the active protein ingredient. A combination of the methods described below may be employed to achieve that goal.

3.1. Chromatographic Methods

As with smaller chemical entities, chromatography is a powerful tool in evaluating both the purity and degradation profile of proteins. The most common chromatographic methods used to profile proteins are reverse-

phase high-pressure liquid chromography (RP-HPLC) and size-exclusion chromatography (SEC).

RP-HPLC relies on a relatively nonpolar stationary phase in conjunction with an aqueous-based polar mobile phase. For large protein molecules, C4 or C8 alkanes are bonded to silica-based or polymeric supports as the stationary phase. Mobile phases typically consist of aqueous acetonitrile gradients with trifluoroacetic acid constant at 0.1%. Phosphate or TRIS buffers may be used to adjust pH for optimal separation. RP-HPLC has been applied extensively to the characterization of insulin formulations (Welinder and Andresen, 1982). Kroeff and Chance (1982) describe the use of RP-HPLC as a definitive identity test for insulin, as a means of monitoring the levels of insulin-related substances, and as a method for evaluating stability samples. Furthermore, for insulin formulations, a correlation was established between the assay value obtained by RP-HPLC and the biological potency. Geigert *et al.* (1988) used RP-HPLC as part of the stability evaluation of recombinant human fibroblast interferon in a formulation containing sodium dodecyl sulfate (SDS). Riggin *et al.* (1987) describe an isocratic RP-HPLC method that demonstrated the capability of distinguishing both desamido and sulfoxide derivatives of human growth hormone.

Size exclusion chromatography can provide information as to the levels of aggregation and fragmentation in a protein pharmaceutical. Watson and Kenney (1988) used size exclusion chromatography to investigate the disappearance of monomer and appearance of aggregates of interferon-γ and interleukin-2 (IL-2) after storage at elevated temperature, after mechanical agitation, and following rapid freeze thaw.

3.2. Optical Techniques

Quantitation of the manner in which proteins absorb, emit, and scatter light provides valuable information about the protein's conformation in a particular formulation and, importantly, about the tendency of the protein to aggregate.

The intensity of scattered light depends on a number of measurable quantities and can be expressed as a function of the number of centers of scattering per unit volume (Bier, 1957). Light scattering can therefore be used as a measure of the number of protein aggregates in a formulation. Brems (1988) measured turbidity at 450 nm as part of an evaluation of the solubility of different folding conformers of bovine growth hormone. Mulkerrin and Wetzel (1989) also used light-scattering measurements, at a nonabsorbing wavelength, to study the effect of pH on thermal denaturation

of α interferons. One of the limitations is that the turbidity is only linearly related to the extent of aggregation when the particle size is small compared with the wavelength of light (Bier, 1957; Sonntag and Strenge, 1970). When the precipitate in a protein formulation consists of large macroscopic particles, a visual ranking system or the use of light microscopy may be more valuable in assessing the physical stability of the formulation (Sonntag and Strenge, 1970; Quinn and Andrade, 1983; Lougheed *et al.*, 1983).

Optical spectroscopic techniques used to evaluate protein pharmaceuticals include ultraviolet and visible absorption spectroscopy, optical rotatory dispersion (ORD), and circular dichroism (CD), fluorescence, infrared, and Raman spectroscopy. Cantor and Timasheff (1982) have reviewed these techniques extensively and detailed their utility in the evaluation of proteins. Of the visible spectroscopic techniques, CD spectroscopy has seen the more rapid and dramatic growth. The far UV circular dichroism spectrum of a protein is a direct reflection of its secondary structure (Hennessey and Johnson, 1981; Johnson, 1985, 1988). An asymmetric molecule, such as a protein macromolecule, exhibits CD because it absorbs circularly polarized light of one rotation differently from circularly polarized light of the other rotation. The technique is therefore useful in determining changes in secondary structure as a function of stability, thermal treatment, or freeze thaw. Brems *et al.* (1987) used far-ultraviolet CD to study the helical structure of isolated fragments of bovine growth hormone. The amount of helix was found to be dependent on pH and peptide concentration. Johnson (1985) provides an excellent review of the technique and numerous examples of the application of CD to study protein denaturation. Manning (1989) has also reviewed this technique and discussed the potential problems in the use of CD for quantitative estimates of secondary structure.

3.3. Electrophoresis

Electrophoretic techniques are based on differences in the electrically induced migration of a protein in a sievelike gel based on the molecule's size and/or net charge. Differential migration patterns may result between the parent protein and degradation products with denaturation, which may result in a change in molecular size due to aggregation, or chemical modification, which might also affect the molecule's net charge at a particular pH. The two techniques used most often as part of a protein formulation stability program are sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and isoelectric focusing (IEF). SDS–PAGE involves denaturation of the protein sample with the SDS anionic detergent followed by

electrophoresis through the polyacrylamide gel support. Smaller protein molecules migrate faster than larger molecules. SDS–PAGE may be carried out under reducing conditions or nonreducing conditions. Nonreducing conditions are often used to assess the state of aggregation of the protein formulation. A comparison of the reduced versus nonreduced samples can be used to identify covalently cross-linked aggregation. IEF uses a pH gradient to separate molecules based on their isoelectric points. As the isoelectric point is dependent on the charged functional groups in the amino acid sequence, the migration will be influenced by such processes as deamidation or the degree of glycosylation of the molecule. Electrophoretic techniques have been included in the stability evaluation of methionyl human growth hormone (Jones and O'Connor, 1982), tumor necrosis factor preparations (Geigert *et al.*, 1987), and human interferon β (Geigert *et al.*, 1988).

3.4. Potency–Activity Assays

Under the current regulatory guidelines, protein pharmaceuticals produced using recombinant DNA technology and hybridoma technology are classified as biologicals. As such, formulations containing these agents must be assayed using a method that is capable of directly monitoring the biological potency of the protein as specified in the Code of Federal Regulations 21, 610.10. It is reasoned that this is ultimately the only means of assuring that the biological conformation, and therefore the efficacy, has been maintained over the shelf life of the product.

In order to substitute a physicochemical method for the biological potency assay, a correlation must exist between the results of the two tests. In the case of insulin, data have been accumulated to demonstrate such correlations (Pingel *et al.*, 1982; Kroeff and Chance, 1982; Sjodin *et al.*, 1982). For many more recent recombinantly derived proteins, the stability evaluation incorporates a potency test in addition to other chemical test methods such as those described above. Potency assays may be animal model assays, such as the hypoglycemic response in rabbits with insulin; cell culture based, such as the cytopathological effect assay for interferons; or *in vitro* biochemical tests, such as the clot lysis assay used for tissue plasminogen activator.

3.5. Other Techniques

Several other techniques for studying the effect of heat, shear, surface phenomena, and solvent additions on the pro[illegible]native state have been used. Horne *et al.* (1971) used cloud point and synthetic polymers as model

proteins. The cloud point likely occurs from the dissolution of a molecule's hydration layer, exposing the hydrophobic interior and resulting in precipitation of the molecule. The effect of electrolytes, alcohols, sugars, and urea on polyvinyl methylether (a model polymer) was measured, and the author suggests that these effects may be predictive of what happens to protein stability in an aqueous media. Gratzer and Beaven (1969) studied a protein denaturant's effect on hydrophobic bond breakage by measuring the denaturant's ability to effect the critical micellar concentration (CMC) of a detergent. The UV absorption spectrum of the detergent is perturbed upon micelle formation. Sucrose caused no effect on CMC and stabilizes proteins, while urea disrupted micelle formation and is known to denature proteins. Thus, this parallel effect of solutes on CMC and protein structure allows some predictability of excipient effects.

Ross (1974) and Ross *et al.* (1984) used scanning microcalorimetry to measure energies of transitions in solution. This can be applied to investigation of transitions in proteins as a function of temperature, as well as stabilizing effects of excipients. Yu and Finlayson (1984) studied effects of stabilizers on human serum albumin and found that the transition temperature was higher in the presence of stabilizers. A number of other groups have used thermal analysis as a tool to study denaturation (Back *et al.*, 1979; Uedaira and Uedaira, 1980; Fujita and Noda, 1981; Gekko, 1982).

Busby and Ingham (1984, 1987) and Busby *et al.* (1981) utilized fluorescent probes and fluorescence spectroscopy to examine stabilization of antithrombin III by heparin and lyotropic anions and sugar derivatives.

4. FORMULATION DEVELOPMENT

Understanding the principles of protein stabilization and armed with the methods to measure changes, the formulation scientist can then use chemical or physical methods to provide a marketable dosage form with acceptable shelf life. Traditionally, proteins have been formulated as parenteral (injectable) products, although more recently there has been a move toward alternate routes of administration such as rectal, nasal, oral, and aerosol for inhalation. This will lead to a demand for dosage forms with increased complexity in their formulations beyond the need to be sterile, isotonic, near-neutral pH, and preserved. The goal of every good formulation is to provide for an easily administered, efficacious product with adequate stability to provide a reasonable shelf life for marketing. Simplicity of formulation is desirable because each additional excipient increases the potential for ir[illegible]tion with the drug or for the appearance of inherent instability. Although not covered in detail in this chapter, the choice of container

and closure or delivery device is critical because of the propensity for these molecules to adsorb to surfaces or be destabilized by extractables from the package material. This is reviewed by Wang and Chien (1984).

4.1. Stabilization Techniques Using Chemical Additives

Historically, a number of different molecular types have been used as stabilizers in protein formulations, including sugars, amino acids, surfactants, and fatty acids.

4.1.1. Proteins

Protein drugs are often so potent that only dilute concentrations are needed. This creates a problem in that proteins are often more unstable in dilute solution than when concentrated. They are also more likely to show the surface adsorption phenomenon. For this reason, other proteins, most often serum albumin, are added. Albumin is a good choice because it is innately very stable and soluble. A number of patents discuss the use of albumin as a stabilizer (Wang and Hanson, 1988). Many studies have shown that albumin can prevent the adsorptive loss of insulin in plastic bags or tubes and glassware (Wang and Chien, 1984). Caution should be exercised, however, because albumin also binds molecules. Law *et al.* (1983) reported increased loss of thyroid hormone on a filter at albumin concentrations of 2.5–5%.

Albumin can act as a substitute for naturally occurring protein stabilizers. Wolf *et al.* (1972) reported that adenyltransferase in the cell was bound to a second protein with stabilizing properties. As this was removed during purification, albumin could be substituted and bound to the same sites, but with a lower affinity. Hsu *et al.* (1982) reported that the natural conformation of rabbit muscle creatine phosphokinase was stabilized by albumin, and Wiseman and Williams (1971) postulated that albumin protected alcohol dehydrogenase. The disadvantage of using albumin or other proteins in a formulation is that you have clearly reduced the specific activity of the molecule after much effort has gone into purifying it. The addition of each protein excipient carries the potential of adding impurities to the formulation if the excipient itself is not 100% pure.

4.1.2. Amino Acids

Amino acids have been found to have multiple effects in protein formulations. As seen with proteins, amino acids reduce the surface adsorption.

Mizutani (1981) reported that acidic amino acids, not aliphatic or aromatic, would inhibit adsorption of hemoglobin, serum globulin, and lysozyme to silicone-coated glass.

Amino acids also prevent aggregation reactions. Gamma globulins in solution isolated from human plasma tend to aggregate and precipitate. Coval (1978, 1979) used organic acids, glycine, and, when lyophilized, mannitol to achieve an unaggregated solution with good stability. Also, Takagi and Matsuo (1980) used arginine and lysine as stabilizers of an intravenous immune globulin preparation. Ornithine, aspartic acid, glutamic acid, alanine, and glycine did not stabilize. Insulin solutions tend to aggregate. Bringer *et al.* (1981) found that glutamic acid and aspartic acid at low pH prevented precipitate formation even after shaking for four days. Quinn and Andrade (1983) reported similar results with lysine alone or in combination with EDTA. Chelation of zinc was postulated as a mechanism. Recently, it has become the practice to heat therapeutic products isolated from human blood to 60 °C for 10 hr to inactivate possible viral contamination. The activity of the coagulation protein, factor XIII, is destroyed by this heating. However, Fukushima *et al.* (1981) has reported that neutral amino acids protect the activity as do sugars and carboxylic acids.

Amino acids have been reported to stabilize plasmin solutions, plasmin being a proteolytic enzyme that lyses fibrin in the blood. Plasmin that is isolated from plasma is unstable in a neutral solution, demonstrated by a rapid loss of proteolytic activity at 37 °C. However, Jensen (1976) reported that various amino acids stabilized the plasmin activity and seemed to improve solubility, in that solutions of plasmin in water would become turbid due to precipitation even at 0.01 U/ml but remain clear up to 25 U/ml in the presence of amino acids. Aliphatic amino monocarboxylic acids were the most effective, and the stabilization increased with increasing carbon chain length. Branched α-amino acids were also effective, as were amino acids whose amino group was substituted to be more basic. For example, guanidine acetic acid and creatine are better than glycine and valine. Dipeptides (glycyl glycine) were effective and supported the theory that the amino group be as far as possible from the carboxylic group of the amino acid. Patents that cover use of amino acids as stabilizers are listed in Wang and Hanson (1988).

4.1.3. Phospholipids and Fatty Acids

The stabilizing effect of fatty acids and phospholipids was recognized early. Boyer *et al.* (1946) measured the thermal stabilization of serum albumin by fatty acids and found straight-chain acids of seven to eight carbons had maximum effect. Shrake *et al.* (1984), Yu and Finlayson (1984), and Ross (1974), using differential scanning calorimetry, found caprylate and *N*-acetyl-DL-tryptophanate stabilized albumin to heating

The coagulation factor VIII, when incubated with phospholipid, has two to three times higher coagulant activity (Broden *et al.*, 1983). Factor VIIIc, normally complexed to von Willebrand factor in circulation, binds phospholipid in the absence of von Willebrand factor and is more stable (Andersson and Brown, 1981). Lajmanovich *et al.* (1981) found this was dependent on which phospholipid was used. Lollar *et al.* (1984) reported similar results with porcine factors VIII and IXa. Moss *et al.* (1984) found lysophosphalidyl cholines, but not related phospholipids, activate and stabilize NAD–arginine ADP ribosyltransferase.

4.1.4. Surfactants

While surfactants are more usually associated with denaturing of protein, they have been found to be of some use to formulators in certain circumstances. Gibbs *et al.* (1952) demonstrated that aliphatic amines above their CMCs prevented flocculation of denatured egg albumin. Others (Markus and Karush, 1957; Takeda and Hizukuri, 1972; Stewart *et al.*, 1974, 1975; Mogensen and Cantell, 1974) have reported the activation or stabilization of proteins by surfactants. The marketed product Activase®, a form of tissue plasminogen activator, lists 0.4% Polysorbate 80 as an excipient in the 1990 *Physicians' Desk Reference.*

Proteins tend to concentrate at interfaces. A monolayer of protein will form at an air–water interface but will not redissolve because of irreversible unfolding (Adamson, 1960). This may account for turbidity observed in some protein solutions after shaking (Henson *et al.*, 1970). To prevent denaturation at surfaces, surfactants such as poloxamer 188 (Pluronic 68) or polysorbate have been used in injectable formulations (Coval, 1979). This author, when formulating a large, globular plasma protein for lyophilization, found that low levels of Polysorbate 80 were required to achieve reconstitution.

Use of any surfactant in a formulation must be considered carefully and be restricted to the lowest levels possible due to the possible toxicity and hypersensitivity reactions. This is especially true of pediatric formulations, since the problems seen in children taking vitamin E preparations were thought to be caused by the higher levels of Polysorbate 80. Some products containing polyoxyethylated castor oil have been removed from the market.

4.1.5. Metals

There is extensive literature on the need for metals such as calcium for activity or stability of an enzyme or other protein molecule, (Weiss, 1965;

Ahmed *et al.*, 1975; Gomez *et al.*, 1977; Marguerie, 1977; Siegel and Martin, 1982; Mikaelsson *et al.*, 1983). This author found that the level of $MgCl_2$ remaining in a product after gradient elution from a column in concentrations of 0 to 0.5 M was linearly related to the moisture level in the lyophilized cake following freeze-drying with the same cycle. At higher than 0.3 M $MgCl_2$, melt back occurred.

4.1.6. Polyols

Polyols are defined as any substance with multiple hydroxyl groups, including polyhydric alcohols (sorbitol, mannitol, glycerol) and carbohydrates (keto or aldehyde sugars). Enzymologists and formulation scientists have routinely used sugars or alcohols to stabilize enzymes in storage or protein drugs, especially during lyophilization, without perhaps understanding what mechanism was operating.

Gamma globulins are particularly unstable in solution and exhibit "shedding," the formation of visible, insoluble protein particles. Lundblad *et al.* (1980), Fernandes and Lundblad (1980, 1981) showed that maltose prevented this reaction. They also demonstrated that other plasma proteins (plasminogen, antithrombin III) were stabilized against heat inactivation. Miekka *et al.* (1985) demonstrated similar effects with fibronectin, a plasma glycoprotein.

It is the polyhydric alcohols that stabilize and monohydric alcohols that destabilize native conformation, and this effect is concentration dependent (Gerlsma, 1968, 1970; Gerlsma and Sturr, 1972). Back *et al.* (1979) used differential scanning calorimetry to demonstrate similar effects on lysozyme and chymotrypsin, as did others (Oakenfull and Fenwick, 1977; Uedaira and Uedaira, 1980; Gekko, 1982; Andersson and Hahn-Hagerdal, 1987).

While polyols had been used extensively as stabilizers, the mechanism was not always understood. Lee and Timasheff (1981) studied thermal unfolding of three enzymes and suggested the mechanism was due to ordering of water around the protein; the sugar exerts pressure to reduce surface contact between the protein and the solvent. Unfolding increases surface area and increases the volume from which sucrose is excluded, resulting in an unfavorable energetic state. This also allows the hydrophobic groups to be buried inside, away from the water layer. The denaturing effect of monohydric alcohols increases with chain link and is likely caused by interaction with these hydrophobic areas (Bull and Breese, 1978). The mechanisms of stabilization have been further described by Gekko and Timasheff (1981a,b), Arakawa and Timasheff (1982), Privalov and Gill (1988), Miller and Robyt (1984), Monsan and Combes (1984), Busby and Ingham (1984), and Timasheff and Arakawa (1989).

4.1.7. Antioxidants

Disulfide bond formation can be a cause of loss of protein activity that some thiols can inhibit. Shear-induced inactivation of interferon was prevented by glutathione, thioacetic acid, and cysteine. Mercaptoethanol and dimercaptopropanol not only failed to protect unsheared interferon, but inactivated it as well (Cartwright *et al.*, 1977). Dithiothreitol reduced the interchain disulfide of gamma globulin, allowing alkylation and increased biological half-life (Pappenhagen *et al.*, 1975). The antioxidant bisulfite is not suitable for sulfhydryl enzymes, usually decreasing activity (Fujimoto *et al.*, 1983). Pharmaceutical reducing agents have been reviewed by Akers (1982).

4.2. Stabilization Techniques Using Physical Methods

Few proteins have demonstrated adequate stability in solution to provide marketable pharmaceuticals with shelf lives of one year or more. Typically, methods of drying the protein in order to retard degradation are needed. Lyophilization and spray-drying are processes with a long history of use in the pharmaceutical and food industries for the stabilization of otherwise easily degraded substances. Both technologies may be used to dehydrate heat-sensitive molecules and so prevent degradation observed when proteins are in solution.

4.2.1. Lyophilization

Lyophilization is the most common method of drying proteins for pharmaceutical applications. The lyophilization process is divided into three stages: freezing, primary drying, and secondary drying. During the freezing stage ice crystals form, causing the unfrozen fraction of the formulation to become increasingly concentrated. Freezing continues until the water and solutes are completely frozen. The primary drying phase involves the sublimation of ice from the product. The drying is driven by reducing the pressure in the chamber and supplying heat to the product. By drying the product at reduced pressure, the removal of water can be achieved in reasonable times at relatively low (subambient) temperatures. During the secondary drying stage, desorption of moisture from the product occurs. The temperature and extent of secondary drying influences the final moisture content of the product. This in turn affects the long-term stability of the product (Pristoupil *et al.*, 1985; Hageman, 1988).

The need to resort to lyophilization to stabil[illegible] protein affects the

approach used to formulate the product. The choice of excipients is influenced not only by the desire to stabilize the product, but also by the ability to prepare a pharmaceutically acceptable and elegant lyophilized plug. Excipient selection and quantity also influence the time cycle required for lyophilization. Additives may affect melting or collapse temperatures and therefore affect the optimum lyophilization conditions. Furthermore, excipients may be needed to alter the tonicity of the formulation or to control other parameters of a pharmaceutically acceptable parenteral formulation such as its microbiological status through the use of a preservative.

4.2.1a. Formulation Considerations. A typical lyophilized formulation may contain the following classes of excipients:

1. *Bulking Agent.* Often the unit dose amount of protein is low and so each vial may contain very low amounts of total solid. After the lyophilization process and the removal of water, the vial may appear to contain very little product and no lyophilized plug will form. Bulking agents are therefore frequently used to enhance the appearance of the final lyophilized product. Mannitol is a widely used bulking agent that produces an elegant lyophilized cake. It has the further advantage that its concentration in the formulation can be adjusted to alter tonicity. Other polyhydric alcohols such as sorbitol and sugars such as sucrose, dextrose, and dextrans are also used as bulking agents.
2. *Buffer.* Knowledge gained during the preformulation evaluation of the protein as to the solubility and stability as a function of pH is applied to the selection of an appropriate buffering agent to control the pH at its optimal level. The buffer chosen must be pharmaceutically acceptable. If the protein is highly sensitive to changes in pH, the effect of temperature on the pk_a of the buffer selected must be considered. For example, the pk_a of Tris buffer varies by 0.028 units per degree centigrade (Blanchard, 1984), so Tris would be a poor choice in a lyophilized formulation of a protein highly sensitive to pH changes.
3. *Tonicity Modifier.* Parenteral formulations intended for administration by the intramuscular or subcutaneous routes are preferably isotonic with body fluids. As mentioned previously, it may be possible to control tonicity by adjusting the concentration of the bulking agent. In addition to mannitol, dextrose and sodium chloride are commonly used to control tonicity. Where it is not possible or desira[illegible] adjust the tonicity of the formulation prior to lyophiliza-

tion, an alternative approach is to reconstitute the product prior to administration with an isotonic diluent.

4. *Cryoprotectant and Lyoprotectant.* The term "cryoprotectant" is used to describe an excipient that stabilizes the protein to the effects of freezing (Tamiya *et al.*, 1985). The phenomenon of freeze denaturation of proteins has been widely described in the literature (Chilson *et al.*, 1965; Tamiya *et al.*, 1985; Franks *et al.*, 1988). During the freezing stage of the lyophilization process, the concentration of salts can be particularly damaging to labile proteins (Franks, 1989). Carpenter and Crowe (1988) have proposed that the mechanism of cryoprotection afforded to isolated proteins by solutes can be accounted for by the fact that these solutes are preferentially excluded from contact with the protein surface. This proposed mechanism of cryoprotection is the same as the mechanism for stabilizing proteins in solution described by Arakawa and Timasheff (1982) and Lee and Timasheff (1981). Therefore, solutes selected for cryoprotection are often the same compounds that have been found to stabilize proteins against solution induced perturbations.

   Cryoprotection has been associated with a wide range of diverse compounds including sugars, polyols, amino acids, methylamines, and lyotropic salts (Doebbler, 1966; Crowe *et al.*, 1987; Carpenter and Crowe, 1988; Gray, 1988). Many sugars that are used as bulking agents, such as mannitol, sorbitol, and sucrose, are also cryoprotective agents and so serve a dual role in the formulation. Nonreducing sugars, such as sucrose or trehalose, are preferred to the reducing sugars, such as maltose or lactose. This is because of the propensity of the reducing sugars to participate in protein browning via the Maillard reaction (Carpenter *et al.*, 1987a).

   A lyoprotectant has recently been defined as an agent that stabilizes and prevents degradation of a macromolecule during both freeze-drying and subsequent storage (Townsend and DeLuca, 1988). They found that sucrose, Ficoll 70, and polyvinylpyrollidone offered lyoprotection by preserving the enzymatic activity of lyophilized RNase.

4.2.1b. Optimization of the Lyophilization Cycle. Protein pharmaceuticals represent very high-value products, and so it is especially important to develop a full understanding of the process to which the product is subjected during freeze-drying. Optimization of the lyophilization cycle is critical from an economic perspective in reducing process run [illegible] and preventing loss

of product due to melt back or collapse and the consequent loss of cake structure. The freezing and drying conditions employed may also have profound effects on the long-term stability of the final product. Cycle development depends on an understanding of the thermal properties of the formulation. The parameters of importance include the freezing temperature of the formulation, the temperature at which the frozen formulation will melt or collapse, and the temperature above which the product will rapidly degrade. For a formulation that forms a eutectic mixture upon cooling, the critical temperature to be determined is the melting point of the eutectic (T_e). For formulations that do not crystallize but form an amorphous glass upon cooling, the glass transition temperature is the point at which the physical state of the glass becomes rubbery. If lyophilization begins above the glass-transition temperature, collapse of the cake will occur. The moisture level and physical properties (amorphous or crystalline) required to achieve long-term stability must also be considered in developing the lyophilization cycle.

Methods used to characterize the thermal properties of formulations for lyophilization include thermal analysis (DSC and DTA), resistivity measurements, and microscopy (MacKenzie, 1965, 1975, 1977; Gatlin and DeLuca, 1980; Jennings, 1980; Williams and Polli, 1984). Application of these techniques and careful interpretation of the results permits the determination of the maximum primary drying temperature the formulation can tolerate without eutectic melt or collapse, resulting in loss of cake integrity and an unacceptable product. During the secondary drying stage, the maximal shelf temperature to which the product can be raised must be determined. The goal is to bring about the desorption of water at a maximal rate but at a temperature that will not cause product decomposition. The duration of the secondary drying phase affects the final moisture content of the product.

The final moisture content attained is also influenced by the morphology of the lyophilized cake. The amorphous or crystalline nature of the cake depends on a number of factors including the rate of freezing and the degree of supercooling (Jennings, 1980), the formulation excipients (Korey and Schwartz, 1989), and thermal treatment of the product during freeze-drying (Gatlin and DeLuca, 1980). Gatlin and DeLuca (1980) reported that solutions of cefazolin sodium and nafcillin sodium undergo solid-state transitions from the amorphous to the crystalline form in the frozen phase when thermally treated. The physical state of the product affects the mass transfer of water vapor from the lyophilized cake. The drying rate is higher for a crystalline versus an amorphous cake (Gatlin and DeLuca, 1980; Korey and Schwartz, 1989). However, an amorphous cake structure may be preferable in some instances to achieve more rapid dissolution, and the amorphous physical state may be associated with the lyoprotective effect of several compounds (Tow[illegible]d and DeLuca, 1988). This may be rationalized on the

basis of the potential physical molecular interaction of the protein and excipient when in an amorphous configuration, compared to the physical isolation associated with crystalline solids.

A full account of the strategies and techniques used for lyophilization cycle development is beyond the scope of this chapter. It is important, however, to consider carefully the role of the formulation excipients in affecting thermal and physical properties and therefore directly impacting the lyophilization cycle used. In certain lyophilized formulations the sole purpose of an ingredient in the formulation may be to alter the freezing and melting characteristics of the formulation in order to accommodate a practical and economical lyophilization cycle.

4.2.2. Spray-Drying

Spray-drying provides an alternative to freeze-drying as a process that is capable of drying thermally labile compounds such as protein molecules. It has been used extensively in processing foods. In the pharmaceutical industry, spray-drying is used to dry heat-sensitive pharmaceuticals, to change the physical form of materials for use in tablet and capsule manufacture, and to encapsulate solid and liquid particles (Rankell *et al.*, 1986). In the spray-drying process, a liquid feed stream is first atomized for maximal air spray contact. The particles are then dried in the air stream in seconds due to the high surface area contact with the drying gas. The major advantage of spray-drying compared to lyophilization is that the drying process can be used to manipulate the shape and size of the dried product. Spray-drying can produce spherical particles that have good flow properties, and the process can be optimized to produce particles of a range of sizes dependent on the particular application.

A recent European patent application by Steber *et al.* (1988) describes the use of spray-drying and spray-cooling to produce sustained-release microspheres of biologically active proteins for parenteral administration to animals. Bovine growth hormone was prepared in the size range suitable for incorporation into microspheres by spray-drying. A mixture of the active protein ingredient and additives in a molten mix of fat and wax is then sprayed through an air–liquid spray nozzle equipped with a heated jacket to maintain the incoming air and the molten phase above the melting point. Microspheres form as the molten droplets cool and are collected on a series of sieves in the desired size range of about 45–180 μm. Sustained-release microspheres of bovine growth hormone prepared in this manner were used to increase weight gain and milk production in dairy cattle.

Several studies have shown that additives co[illegible]nly used to protect proteins during freeze-drying are also useful protectants in a spray-drying process. Labrude *et al.* (1989) found that sucrose had a protective effect on both the spray-drying and lyophilization of oxyhemoglobin. In addition, Carpenter *et al.* (1987b) have demonstrated that compounds such as sucrose and trehalose, which have been shown to protect proteins during freezing, also protected the model enzyme phosphofructokinase from the denaturing effects of air-drying.

The main limitations with spray-drying are the high product losses often encountered and the difficulties in attaining low moisture levels in the product (Labrude *et al.*, 1989). However, as more therapeutic proteins are developed, there is a wider interest in exploring more sophisticated dosage forms and routes for their administration. As has been demonstrated for the sustained-release bovine growth hormone described above, spray-drying offers a means of processing proteins to produce easily handled bulk powders, which may then be processed further for a wide range of pharmaceutical applications.

5. CONCLUSION

It is hoped from this brief review of the literature that those beginning formulation of a protein or peptide drug are aware that there are a number of excipients and mechanisms, both chemical and physical, available by which stable, marketable therapeutic agents can be obtained. Knowledge of the protein or peptide, including amino acid sequence, structure, cellular environment, and cofactors, will allow prediction of what stabilizers will be needed. Newer and more sophisticated analytical methodologies are available to judge the success of these stabilizers in the formulation. It is critical to keep a formulation as simple as possible and to understand the rationale for each excipient added.

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Stability of Protein Pharmaceuticals

Part B
In Vivo Pathways of Degradation and Strategies for Protein Stabilization

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and
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(54) Stabile Transglutaminasepräparate und Verfahren zu ihrer Herstellung

(57) Die vorliegende Erfindung betrifft stabile Zubereitungsformen einer Transglutaminase, beispielsweise Faktor XIII, die nach Lyophilisieren gut und ohne Trübung löslich sind und die gereinigte Transglutaminase sowie D- und/oder L-Aminosäuren, außer Glycin und Arginin, deren Salze, Derivate und Homologe, Dimere oder Oligomere davon oder Mischungen davon und/oder Zucker oder Zuckeralkohole, gegebenenfalls in Kombination mit oberflächenaktiven Agentien und/oder reduzierenden Agentien, enthalten. Die Erfindung betrifft außerdem Verfahren zur Herstellung stabiler Proteinpräparationen sowie die Verwendung der beschriebenen stabilen Zubereitungsformen zur Herstellung von Arzneimitteln, die beispielsweise zur Behandlung von durch F XIII-Mangel charakterisierten Erkrankungen geeignet sind.

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Beschreibung

Gegenstand der vorliegenden Erfindung sind stabile Transglutaminasepräparate, insbesondere stabile Faktor XIII-Präparate, und Verfahren zu ihrer Herstellung.

Faktor XIII (F XIII, Fibrin-stabilisierender Faktor), eine in Plasma, Plättchen und Monozyten/Makrophagen als Proenzym vorkommende Transglutaminase, ist u.a. für eine ungestörte Blutgerinnung und Wundheilung von Bedeutung. F XIII wird in Form von frisch gefrorenem Plasma, isoliert aus Plazenta oder Plasma therapeutisch schon über Jahre hinweg mit gutem Erfolg für die Therapie des Faktor XIII-Mangels eingesetzt. Mittlerweile ist auch die rekombinante Herstellung von Faktor XIII (rF XIII) möglich.

Die kommerziell angebotenen, gereinigten oder teilgereinigten Transglutaminase- bzw. F XIII-Präparate enthalten zugesetzte Stabilisatoren wie humanes Serumalbumin (HSA), was aber in Hinblick auf die damit verbundene Verminderung der spezifischen Aktivität, die zusätzliche Proteinbelastung bzw. potentielle Immunogenität sowie die Beeinträchtigung der Reinheitsbeurteilung für Proteinpräparate von Nachteil ist. Besonders bei hochreinen Proteinen (wie z.B. rekombinanten Proteinen) ist es wünschenswert, die erreichte Reinheit nicht wieder durch Zusatz von Fremdproteinen zu reduzieren. Ferner ist durch den Zusatz von z.B. Albumin auch eine potentielle Belastung mit Virusantigenen gegeben.

Therapeutisch einsetzbare Proteinpräparate müssen in aller Regel über einen längeren Zeitraum hinsichtlich ihrer Zusammensetzung und Aktivität stabil sein. Da dies in Lösung nur selten erreichbar ist, werden solche Produkte häufig getrocknet in den Handel gebracht. Zur Trocknung solcher Produkte ist die schonende Gefriertrocknung die Methode der Wahl. Doch auch bei dieser Methode werden nur unter bestimmten Voraussetzungen stabile Präparationen erhalten, die die Anforderungen an Integrität und Haltbarkeit erfüllen.

Da die Gefriertrocknung von z.B. unstabilisierten Transglutaminase-Lösungen zu einem starken Aktivitätsabfall und zu erheblichen Trübungen führt, sind z.B. für gereinigte F XIII-Präparate bisher Formulierungen auf Basis von Albumin mit mehr oder weniger hohen Konzentrationen von Salzen beschrieben worden (DE-PS 2063 070, JP 53/59018). Diese Formulierungen haben aber den bereits beschriebenen Nachteil des Fremdproteinzusatzes mit all seinen Problemen.

Ferner wurde die Gefriertrocknung von rF XIII in Gegenwart von Glycin oder Arginin und nicht-reduzierenden Zuckern beschrieben (WO 93/03147). Hier wurden aber keine Aussagen über die Stabilität und Löslichkeit bzw. die Klarheit des rekonstituierten Lyophilisates gemacht. Auch wurde das erhaltene Produkt bei -20°C gelagert, vermutlich um aufgrund einer unzureichenden Stabilität bei 4°C das Material stabil zu halten.

Der vorliegenden Erfindung liegt daher die Aufgabe zugrunde, durch eine geeignete Formulierung für Transglutaminasen, insbesondere für F XIII, als lokal (z.B. auch topisch) oder parenteral applizierbares Protein ein bei 2-8°C (oder höher) stabiles, lagerfähiges Produkt zu erhalten, bei dem auf den Zusatz von z.B. HSA verzichtet werden kann. Ferner sollte das Lyophilisat gut löslich sein und nach dem Einlösen eine klare Lösung ohne Trübungen ergeben, die weiterhin noch eine ausreichende Stabilität besitzen sollte.

Diese Aufgabe wurde durch die Bereitstellung von stabilen Zubereitungsformen für Transglutaminasepräparate unter Verwendung bestimmter Stabilisatoren bzw. Gemische davon, wie sie nachfolgend näher beschrieben sind, und von Verfahren zu ihrer Herstellung gelöst. Die Erfindung betrifft somit eine stabile Zubereitungsform einer Transglutaminase, die nach Lyophilisieren gut und ohne Trübung löslich ist, enthaltend die gereinigte Transglutaminase sowie D- und/oder L-Aminosäuren, außer Glycin und Arginin, deren Salze, Derivate und Homologe, Dimere oder Oligomere davon oder Mischungen davon und/oder Zucker oder Zuckeralkohole, gegebenenfalls in Kombination mit ober-flächenaktiven Agentien und/oder reduzierenden Agentien.

Die beispielhaften Ausführungen zeigen dies anhand von rekombinantem Faktor XIII bzw. des aus Plazenta oder Plasma isolierten F XIII, sind jedoch nicht darauf beschränkt. In einer bevorzugten Ausführungsform betrifft die vorliegende Erfindung somit stabile Zubereitungsformen von Faktor XIII, biologisch aktiven Fragmenten, Derivaten oder Mutteinen davon.

In einer besonders bevorzugten Ausführungsform handelt es sich bei den stabilen Zubereitungsformen um F XIII aus Plasma, Plazenta, Thrombozyten, Makrophagen/Monozyten oder rekombinanten F XIII enthaltende Zubereitungsformen.

Die erfindungsgemäßen Untersuchungen lassen sich im wesentlichen in zwei Bereiche aufteilen: (a) Untersuchungen zur eigentlichen Gefriertrocknung und (b) zur anschließenden Lagerstabilität.

Für die Untersuchungen wurde rF XIII (Metzner et al., in J. McDonagh, R. Seitz, R. Egbring: "Factor XIII", 87 - 93, Schattauer (1993)), sowie plazentärer und plasmatischer F XIII eingesetzt (Karges u. Rapp, in J. McDonagh, R. Seitz, R. Egbring: "Factor XIII", 66 - 76, Schattauer (1993)).

Die Gefriertrocknungsversuche wurden in handelsüblichen Anlagen unter Verwendung von Glasfläschchen ohne bzw. mit silikonisierter Oberfläche durchgeführt.

(a) Gefriertrocknung:

Bei dem Vergleich verschiedener Additive wurde überraschenderweise gefunden, daß bei Verwendung von bestimmten Zusätzen aus der Gruppe der D- und/oder L-Aminosäuren, deren Salze, Derivate oder Homologe die Aktivität und Löslichkeit des F XIII bei der Gefriertrocknung teilweise sehr gut erhalten werden kann (Tabelle I).

In einer weiteren bevorzugten Ausführungsform betrifft die vorliegende Erfindung daher stabile Zubereitungsformen einer Transglutaminase, die die Aminosäuren His, Glu, Met, Thr, Lys, Ala, Ile, Cys, deren Salze, Derivate, Homologe, Dimere oder Oligomere davon, oder Mischungen davon enthalten.

Speziell die Aminosäuren His und Glu zeigten überraschenderweise bereits ohne weitere Zusätze eine gute Stabilisierung bei der Gefriertrocknung. Bei alleinigem Einsatz von Aminosäuren wie z.B. Gly, Met oder Ala kam es hingegen bei der Gefriertrocknung zu einem deutlichen Aktivitätsabfall (Tabelle I).

Der alleinige Einsatz von Zuckern oder Zuckeralkoholen ergab teilweise bereits eine gute Stabilisierung im Verlauf der Gefriertrocknung (Tabelle I). Besonders Zucker oder Zuckeralkohole wie Saccharose, Trehalose, Laktose, Maltose, Sorbit, Mannit u.a. zeigten positive Ergebnisse. Der Aktivitätsabfall bei der Gefriertrocknung mit Aminosäuren als Zusatz, die alleine nicht ausreichend wirksam waren (wie z.B. Met, Ala, u.a.), läßt sich durch Kombination mit Zuckern oder Zuckeralkoholen stark reduzieren (Tabelle I).

In einer weiteren Ausführungsform betrifft die vorliegende Erfindung deshalb stabile Zubereitungsformen einer Transglutaminase, die die Zucker oder Zuckeralkohole Saccharose, Lactose, Trehalose, Maltose, Sorbit oder Mannit, deren Derivate, Homologe oder Mischungen davon enthalten.

Der alleinige Einsatz von Puffersubstanzen wie Tris oder Phosphat führte zu keiner nennenswerten Stabilisierung. Allerdings führte der Einsatz von Boraten zu einer deutlichen Stabilisierung im Verlauf der Gefriertrocknung (Tabelle I).

Ein bei Einlösen des Lyophilisates teilweise auftretender, geringfügiger Proteinniederschlag ließ sich durch Einsatz von oberflächenaktiven Stoffen wie Tween 80 oder Tween 20, Polyethylenglykol (PEG) mit Molekulargewichten zwischen 1000 und 35000 Da, Cetylalkohol, Polyvinylpyrrolidon (PVP), Polyvinylalkohol (PVA), Lanolinalkohol, Sorbitanmonooleat u.a. ohne Aktivitätsverlust des F XIII bei der Gefriertrocknung verhindern (Tabelle II u. III).

In einer weiteren bevorzugten Ausführungsform enthalten die stabilen Zubereitungsformen als oberflächenaktiven Stoff somit Tween 80, Tween 20, PEG, Cetylalkohol, PVP, PVA, Lanolinalkohol oder Sorbitanmonooleat.

(b) Lagerstabilität:

Ein kritischer Parameter des formulierten Lyophilisates ist die Lagerstabilität, die bei 4°C und bei Raumtemperatur, unter akzelerierten Bedingungen aber auch bei 37°C, bestimmt wurde.

Es zeigte sich, daß für eine gute Lagerstabilität die Kombination von Aminosäuren mit Zuckern, Zuckeralkoholen oder Zuckerderivaten vorteilhaft ist (Tabelle IV). Die Untersuchung verschiedener Zucker ergab, daß Zucker wie Saccharose, Lactose, Trehalose oder Maltose die Aktivität auch bei längerer Lagerzeit unter erhöhter Temperatur stabilisieren, während Zucker wie Glucose oder Fructose als reduzierende Zucker bei 37°C einen langsamen Aktivitätsabfall nicht ausreichend verhindern können (Tabelle V). Auch die Kombination der Aminosäuren His oder Glu, die alleine bereits eine gute Stabilität bedingen, mit Zuckern führte zu einer noch weiter erhöhten Stabilität.

In einer weiteren bevorzugten Ausführungsform enthalten somit die stabilen Zubereitungsformen als Stabilisator Saccharose, Maltose, Trehalose, Lactose, Sorbit oder Mannit, deren Derivate, Homologe oder Mischungen davon, in Kombination mit der Aminosäure His, Glu, Ile und/oder Ala.

Oberflächenaktive Substanzen zeigten im geeigneten Konzentrationsbereich, wie bereits erwähnt, keine negativen Einflüsse auf die Lagerstabilität (Tabelle II u. III).

Obwohl F XIII keine zugänglichen SH-Gruppen aufweist, zeigen SH-Agentien wie Cys, N-Acetylcystein, Thioglycerin oder Glutathion besonders bei erhöhten Temperaturen überraschenderweise einen positiven Einfluß auf die Lagerstabilität von F XIII. Komplexbildner wie z.B. EDTA oder Citrat können dabei zum Schutz der SH-Funktionen zugesetzt werden.

In einer weiteren bevorzugten Ausführungsform enthält die erfindungsgemäße stabile Zubereitungsform einer Transglutaminase daher Cys, N-Acetyl-Cys, Thioglycerin, Natriumsulfid oder Glutathion oder Mischungen davon, ggf. in Anwesenheit eines Komplexbildners.

Sehr gute Ergebnisse bezüglich Aktivitätserhaltung und Löslichkeit des Lyophilisates ließen sich mit ternären oder quaternären Mischungen (Aminosäure(n), Zucker, oberflächenaktive Komponente) erzielen, beispielsweise mit His/Tween/Saccharose-, His/PEG/Saccharose- oder His/Ile/PEG/Saccharose-Mischungen. In einer weiteren bevorzugten Ausführungsform enthält die erfindungsgemäße stabile Zubereitungsform einer Transglutaminase als Zusätze somit außer einer Aminosäure(n) einen Zucker oder Zuckeralkohol und eine oberflächenaktive Substanz, besonders bevorzugt sind dabei die Zusatzmischungen His/Tween 20/Saccharose oder His/Tween 80/Saccharose, His/PEG/Saccharose oder His/Ile/PEG/Saccharose.

In Bezug auf die Lagerstabilität erwiesen sich Mischungen aus Aminosäure(n) und/oder Zucker bzw. Zuckeralkohol, einer oberflächenaktiven Substanz und einem reduzierenden Agens, z.B. Mischungen von Aminosäure(n)/Cys

bzw. N-Acetyl-Cys/PEG/Saccharose, Aminosäure(n)/Thiogly-cerin/PEG/Saccharose und Zucker/ Reduktionsmittel/PEG ebenfalls als ein geeignetes Formulierungssystem, besonders auch bei höheren Temperaturen (siehe Tabelle III und VI). In einer weiteren bevorzugten Ausführungsform enthält daher die erfindungsgemäße Zubereitungsform als Zusatz eine Mischung aus einer Aminosäure(n) und/oder Zucker bzw. Zuckeralkohol, einer oberflächenaktiven Substanz und einem reduzierenden Agens, besonders bevorzugt sind die Mischungen Aminosäure(n)/Cys/PEG/Saccharose, Aminosäure(n) / N-Acetyl-Cys/PEG/Saccharose, Aminosäure(n) / Thioglycerin/PEG/Saccharose und Zucker/Reduktionsmittel/PEG, ggf. in Anwesenheit eines Komplexbildners.

Bei den beschriebenen Formulierungen ist die Konzentration des eingesetzten F XIII in einem weiten Bereich variierbar und liegt vorzugsweise im Bereich von 0,003-50 mg/ml.

Die Konzentrationen der eingesetzten Aminosäuren liegen vorzugsweise in einem Bereich von 0,01-10% (Gew./V.), besonders aber in einem Bereich von 0,1-3% (Gew./V.). Die Zuckerkonzentrationen liegen vorzugsweise bei 0,1-20% (Gew./V.), besonders bevorzugt aber zwischen 0,2-10% (Gew./V.). Oberflächenaktive Komponenten sind vorzugsweise in einem Konzentrationsbereich von 0,00001-5% (Gew./V.) einsetzbar, besonders zwischen 0,0002% und 0,1%. Die Konzentrationen der reduzierenden Agentien liegen vorzugsweise zwischen 0,001% und 2% (Gew./V.), besonders aber zwischen 0,005% und 0,5%.

Für die Lagerstabilität des lyophilisierten Proteins ist auch die Restfeuchte von Bedeutung. Mit den angegebenen Zusätzen lassen sich in einer Schlußphase der Gefriertrocknung die Temperaturen für mehrere Stunden ohne Aktivitätsverlust auf 50-60°C erhöhen, soweit dies für die Reduktion der Restfeuchte notwendig ist.

Für die Gefriertrocknung von Transglutaminasen bzw. F XIII sowie für die anschließende Lagerstabilität sollte der pH-Wert der Lösungen vorzugsweise in einem Bereich von 6-9, besonders bevorzugt zwischen 7 und 8, liegen. Zur Pufferung eignen sich bevorzugt die eingesetzten Aminosäuren, Phosphatpuffer, Boratpuffer oder Trispuffer mit einem pH-Wert im Bereich von 6 bis 9, die ggf. in Kombination mit einem Komplexbildner verwendet werden.

Die vorliegende Erfindung umfaßt auch die Verwendung der oben beschriebenen Stabilisierungszusätze zur Herstellung von stabilen, (ein) Protein(e) enthaltenden Flüssigpräparationen, da die durch die beispielhaft an Transglutaminasen durchgeführten Experimente gewonnenen Ergebnisse auf andere Präparate übertragbar sind.

Die vorliegende Erfindung umfaßt weiterhin ein Verfahren zur Stabilisierung von Proteinen, vorzugsweise Transglutaminasen, bei dem die gereinigten Proteine bzw. das gereinigte Protein, bei dem es sich vorzugsweise um eine Transglutaminase handelt, als Lösung oder Präzipitat mit einer Lösung, die einen oder mehrere der erfindungsgemäßen Zusätze enthält und auf einen für die Stabilität vorteilhaften pH-Bereich eingestellt ist, nach üblicher Vorgehensweise vermischt und danach gefriergetrocknet wird.

Aufgrund der hervorragenden Eigenschaften der nach dem erfindungsgemäßen Verfahren hergestellten stabilisierten Transglutaminasen eignen sich diese sehr gut zur Formulierung eines Arzneimittels.

Die vorliegende Erfindung schließt außerdem die Verwendung der Faktor XIII, biologisch aktive Fragmente, Derivate oder Muteine davon enthaltenden stabilen Zubereitungsformen zur Herstellung eines Arzneimittels zur Behandlung von z.B. durch F XIII-Mangel charakterisierten Krankheiten mit ein.

Die erfindungsgemäßen Arzneimittel können gegebenenfalls mit geeigneten, pharmazeutisch verträglichen, allgemein bekannten Trägern nach bekannten Verfahren formuliert werden. Sie können in geeigneter Dosierung, die vom behandelnden Arzt bestimmt werden kann, verabreicht werden. Die Verabreichung kann auf verschiedenen Wegen erfolgen, z.B. intravenös, intraperitoneal, subkutan, intramuskulär, intradermal oder topisch.

Die Beispiele erläutern die Erfindung.

Beispiel 1

rF XIII, hergestellt durch Expression in Hefezellen und mittels geeigneter Verfahren bis zu einem Gehalt von >98% aufgereinigt, sowie der plazentäre F XIII, ebenfalls in gereinigter Form, wurden als Lösung oder als Präzipitat mit Lösungen der Stabilisatoren versetzt, um zu den angegebenen Aktivitäten zu gelangen. Die F XIII-Lösungen wurden filtriert, in Glasfläschchen abgefüllt und nach einem geeigneten Gefriertrocknungsprogramm getrocknet. Anschließend wurden die Lyophilisate wieder mit aqua dest. auf das ursprüngliche Volumen rekonstituiert. Vor und nach der Gefriertrocknung wurden die F XIII-Aktivitäten bestimmt. Ferner wurde die rekonstituierte Lösung hinsichtlich Trübungen beurteilt.

Die Bestimmung der F XIII-Aktivität erfolgte mit Hilfe des kommerziell verfügbaren Berichrom F XIII^{R} Test Kits.

Die in den Tabellen I, II und III dargestellten Ergebnisse zeigen eindeutig, daß der Zusatz der erfindungsgemäßen stabilisierenden Bestandteile bei der Gefriertrocknung von F XIII notwendig ist und daß durch Zugabe von Aminosäuren oder Zuckern bzw. Aminosäuren und Zuckern die enzymatische Aktivität und die Löslichkeit auch ohne die zusätzliche Verwendung von HSA erhalten werden können. Besonders die Löslichkeit läßt sich durch Zusatz von oberflächenaktiven Agentien teilweise noch verbessern.

Beispiel 2

rF XIII- und Plazenta-F XIII-Lyophilisate, hergestellt wie in Beispiel 1 angegeben, wurden bei 4°C bzw. bei 37°C gelagert und nach verschiedenen Zeiten mit Aqua Injectab. oder mit Kochsalzlösung rekonstituiert, um die verbleibende Enzymaktivität zu bestimmen.

Die in den Tabellen II bis VI dargestellten Ergebnisse zeigen, daß eine ausreichende Langzeitstabilisierung durch die bereits beschriebenen Mischungen, die eine Aminosäure bzw. Aminosäuren und/oder Zucker bzw. Zuckerderivate umfassen, zu erreichen ist. Der Zusatz einer reduzierenden Komponente führt dabei teilweise noch zu einer erhöhten Stabilität, besonders unter akzelerierten Bedingungen.

Tabelle I: Gefriertocknung von FXIII

	Aktivität vor Lyophilis.(%)	Aktivität des Lyophilisates (in % des Ausgangswertes)	Trübung nach Lösen des Lyophilisates
-	100	2	+++
10 mM Tris*HCl, pH 7,4	100	8	+
10 mM Na-Borat pH 8,0	100	68	(-)
1 % L-His, phys.NaCl, pH 7,4	100	39	++
1 % L-His, pH 7,6	100	100	-
1 % L-Arg, pH 7,6	100	96	+/-
1 % L-Gly, pH 7,6	100	41	+/-
1 % L-Ala, pH 7,6	100	67	+/-
1 % L-Glu,pH 7,6	100	88	+
1 % L-Met, pH 7,6	100	10	+++
1 % L-His, 0.1% L-Ile, pH 7,6	100	99	-
1 % Saccharose	100	92	(-)
2,5 % Saccharose	100	91	(-)
5 % Saccharose	100	83	(-)
2,5 % Lactose	100	100	(-)
2,5 % Sorbit	100	92	(-)
2,5 % Trehalose	100	99	(-)
2,5 % Maltose	100	100	(-)
1 % L-His, 2,5 % Sacch., pH 7,6	100	96	(-)
1 % L-Arg, 2,5% Sacch., pH 7,6	100	90	+
1 % L-Ala, 2,5% Sacch., pH 7,6	100	100	-
1 % L-Glu, 2,5 % Sacch., pH 7,6	100	87	+
1 % L-Lys, 2,5 % Sacch., pH 7,6	100	92	(-)
1 % L-Met, 2,5 % Sacch., pH 7,6	100	91	(-)
1 % L-Thr, 2,5 % Sacch., pH 7,6	100	94	+/-

Fortsetzung: Tabelle I

	Aktivität vor Lyophilisation (%)	Aktivität des Lyophilisates (in % des Ausgangswertes)	Trübung nach Lösen des Lyophilisates
1 % L-His, 0,1 % L-Ile, 2,5 % Sacch., pH 7,6	100	94	(-)
1 % L-His, 0,1 % L-Ile, 2,5 % Gluc., pH 7,6	100	85	-
1 % L-His, 0.1 % L-Ile, 2,5 % Lact., pH 7,6	100	99	(-)
1 % L-His, 0,1 % L-Ile, 2,5 % Fruct., pH 7,6	100	92	-
1 % L-His, 0,1 % L-Ile, 2,5 % Sorbit, pH 7,6	100	91	(-)
0, 5 % L-His, 0,1 % L-Ile, 2,5 % Sacch., pH 7,6	100	95	(-)
1 % L-His, 0,1 % L-Ile, 1 % Sacch., pH 7,6	100	93	(-)
1 % L-His, 0,1 % L-Cys, 2,5 % Sacch., pH 7,6	100	107	-

Aktivitätstest: Berichrom FXIII

Trübung : - keine Trübung
(-) minimale Trübung
+/- sehr geringe Trübung
+ leichte, aber sehr deutliche Trübung
++ starke Trübung
+++ sehr starke Trübung

Tabelle II

Einfluß oberflächenaktiver Substanzen auf die Löslichkeit bzw. Stabilität eines FXIII-Lyophilisates										
	Aktivität vor Lyophilisation (E/ml)	Trübung nach Einlösen	Aktivität (E/ml) bei t=							
			0	0,5	1	2	3	6	12	24 Mon.
1 % L-His, 0,1 % L-Ile, 2,5 % Sacch., pH 7,2	153	+	150	144	139		139	143	136	
1 % L-His, 0,1 % L-Ile, 2,5 % Sacch., pH 7,6	154	+	141	151	132		132	139	136	
1 % L-His, 0,1 % L-Ile, 2,5 % Sacch., pH 8,0	152	+	148	143	141		141	138	137	
1 % L-His, 0.1 % L-Ile, 2,5 % Sacch.	129	+	124		122	113	115	111	115	122
1 % L-His, 2,5 % Sacch.	131	(-)	125		126	114	117	108	118	120
1 % L-His, 0,01 % PEG4000, 2,5 % Sacch.	128	-	119		124	114	111	111	114	119
1 % L-His, 0,001 % PEG 4000, 2,5 % Sacch.	130	-	123		125	112	114	112	114	119
1 % L-His, 0,0001%PEG 4000, 2,5% Sacch.	130	-	118		120	107	114	107	107	116
1 % L-His, 0,001 % Tween 20, 2,5 % Sacch.	128	-	123		124	112	114	113	119	119
1 % L-His, 0,0001 % Tween 20, 2,5 % Sacch.	130	-	122		124	112	111	105	112	120
1 % L-His, 10mM Citrat, 2,5 % Sacch.	156	(-)	149	146	138		143	136	147	
1 % L-His, 0,01 % PEG 4000, 2,5 % Sacch.	155	-	147	138	137		140	134	135	
1 % L-His, 0,1 % L-Cys, 2,5 % Sacch.	156	-	156	156	146		148	153	151	
Lagerung der Proben bei +4°C pH 7,6 wenn nicht näher angegeben Aktivitätstest: Berichrom FXIII (E/ml) Trübung : - keine Trübung (-) minimale Trübung + leichte aber sehr deutliche Trübung										

Tabelle III

Lagerung von FXIII Lyophilisat Lagerung der Proben bei Raumtemperatur							
	Aktivtät vor Lagerung (E/ml)	A ktivität (E/ml) bei t =					Trübung nach t =
		1 Monat (E/ml)	3 Monate (E/ml)	6 Monate (E/ml)	9 Monate (E/ml)	12 Monate (E/ml)	1 Monat
1% His/0,001% PEG 4000/2,5% Sacch./pH 7,6	85	99	89	84	86	69	(-)
1% His/0.1% Cystein/0,001% PEG 4000/2,5% Sacch./pH 7,6	92	110	97	103	110	91	-
1% His/0,01% Cystein/0,001% PEG 4000/2,5% Sacch./pH 7,6	87	108	96	98	104	85	-
1% His/0,005% PEG 4000/2,5% Sacch./pH 7,6	99	109	103	99	94	79	-
1% His/0.01% PVP15/2,5% Sacch./ pH 7,6	94	105	96	96	88	78	-
1% His/ 0,1% Ile/ 0,001% PEG 4000/ 2,5% Sacch. /pH 7,6	101	106	92	84	90	69	-
Aktivitätstest: Berichrom FXIII Trübung : - keine Trübung (-) minimale Trübung							

Tabelle IV: Lagerung von rhu FXIII Lyophilisat bei 4°C

	Aktivität vor Lyophilisation (E/ml)	Aktivität (E/ml) des Lyophilisates bei t =							
		0	0,5 Mon.	1 Mon.	3 Mon.	6 Mon.	12 Mon.	18 Mon.	24 Mon.
1 % L-His pH 7,6	125	150	112	95	111	82	100	108	100
1 % L-Gly pH 7,6	125	61	26	48	34	30	29	25	16
1 % L-Glu pH 7,6	125	132	127	116	110	77	114	83	93
1 % L-His, 2,5 % Saccharose, pH 7,6	125	144	112	106	133	97	111	109	94
1 % L-Arg, 2,5 % Saccharose, pH 7,6	125	134	113	106	118	88	113	60	89
1 % L-Ala, 2,5 % Saccharose, pH 7,6	125	150	124	98	114	71	127	102	118
1 % L-Glu, 2,5 % Saccharose, pH 7,6	125	130	108	110	122	84	140	104	90
1% Saccharose	160	153	130	139	136	137	141	129	146
2,5% Saccharose	160	148	127	135	128	135	130	126	141
5% Saccharose	160	130	109	117	113	109	114	107	121
2,5% Lactose	160	163	136	146	137	137	137	131	158
2,5% Sorbit	160	146	122	132	125	122	123	124	148
2,5% Trehalose	160	160	136	142	137	141	136	127	138
2,5% Maltose	160	155	134	139	132	118	139	123	150
1 % L-His, 0,1% L-Ile, pH 7,6	125	148	136	126	93	100	116	105	102
0,5 % L-His, 0,1% L-Ile, 2,5 % Sacch., pH 7,6	125	142	123	113	119	93	116	106	100
1 % L-His, 0,1% L-Ile, 1 % Sacch., pH 7,6	125	139	112	109	127	94	87	108	103
1 % L-His, 0,1 % L-Cys, 2,5 % Sacch., pH 7,6	125	161	135	120	113	91	112	112	101

Aktivitätstest: Berichrom FXIII

Tabelle V

Lagerung von FXIII Lyophilisat bei 37°C						
	Aktivität vor Lyophil. (E/ml)	Aktivität (E/ml) des Lyophilisates bei t =				
		0	1 Mon	3 Mon.	6 Mon.	12 Mon.
1 % L-His, 0,1 % L-Ile, 2,5 % Sacch.pH 7,6	444	394	405	311	300	212
1 % L-His, 0.1 % L-Ile, 2,5 % Glucose pH 7,6	440	373	358	152	34	3
1 % L-His, 0.1 % L-Ile 2,5 % Lactose pH 7,6	424	419	385	322	293	285
1 % L-His, 0.1 % L-Ile 2,5 % Fructose pH 7,6	423	387	385	37	11	3
1 % L-His, 0.1 % L-Ile 2,5 % Sorbit pH 7,6	420	380	298	120	64	21
1 % L-His, 0.1 % L-Ile 2,5 % Maltose pH 7,6	425	366	393	316	293	293
1 % L-His, 0,1 % L-Ile, 0.1 % L-Cys, 2,5 % Saccharose , pH 7,6	413	408	418	350	354	393
1 % L-Glu, 0,1 % L-Ile, 2,5 % Sacchar., pH 7,6	424	395	350	281	301	314
1 % L-Lys, 0,1 % L-Ile, 2,5 % Sacchar., pH 7,6	423	350	403	328	318	333
Aktivitätstest: Berichrom FXIII						

Tabelle VI: Lagerung von FXIII Lyophilisat bei verschiedenen Temperaturen

	Aktivität vor Lyophil. (E/ml)	Aktivität (E/ml) nach versch. Lagerzeiten bei +4°C in Monaten						
		0	0,5 Mon.	1 Mon.	3 Mon.	7 Mon.	12 Mon.	24 Mon.
1 % His/0,001 % PEG/ 2,5 % Sacch./ 0,2 % Cystein/ pH 7,6	112	118	119	117	116	115	122	120
1 % His/0,001 % PEG/ 2,5 % Sacch./0,2 % N-Acetyl-Cystein/ pH 7,6	112	113	123	120	125	118	120	125
1% His/0,001 % PEG/ 2,5 % Sacch./0,2 % Thioglycerin/pH 7,6	119	109	125	122	129	116	129	131
1 % His/ 0,001 % PEG/ 2,5 %Sacch./ pH 7,6	119	109	117	110	118	109	113	116

Lagerung bei Raumtemperatur

		0	0,5 Mon.	1 Mon.	3 Mon.	7 Mon.	12 Mon.	24 Mon.
1 % His/0,001 % PEG/ 2,5 % Sacch,/ 0,2 % Cystein/ pH 7,6	112	118	119	115	116	114	120	112
1 % His/0,001 % PEG/ 2,5 % Sacch./ 0,2 %N-Acetyl-Cystein/pH 7,6	112	113	112	114	120	122	122	120
1 % His/ 0,001 % PEG/ 2,5 % Sacch./ 0,2 % Thioglycerin/pH 7,6	119	109	123	121	120	116	118	116
1 % His/ 0,001 % PEG/ 2,5 % Sacch./ pH 7,6	119	109	117	108	109	105	100	98

Fortsetzung Tabelle VI: Lagerung von FXIII Lyophilisat bei verschiedenen Temperaturen

Lagerung bei + 37°C

		0	0,5 Mon.	1 Mon.	3 Mon.	7 Mon.	12 Mon.	24 Mon.
1 % His/0,001 % PEG/ 2,5 % Sacch,/ 0,2 % Cystein/ pH 7,6	112	118	121	114	119	119	104	89
1 % His/0,001 % PEG/ 2,5 % Sacch./ 0,2 %N-Acetyl-Cystein/pH 7,6	112	113	119	111	112	102	107	89
1 % His/ 0,001 % PEG/ 2,5 % Sacch./ 0,2 % Thioglycerin / pH 7,6	119	109	119	120	113	95	98	85
1 % His/ 0,001 % PEG/ 2,5 % Sacch./ pH 7,6	119	109	107	108	100	85	78	57

Aktivitätstest: Berichrom FXIII

Patentansprüche

1. Stabile Zubereitungsform einer Transglutaminase, die nach Lyophilisieren gut und ohne Trübungen löslich ist, enthaltend die gereinigte Transglutaminase sowie als Zusatz D-und/oder L-Aminosäuren, deren Salze, Derivate und Homologe, Dimere oder Oligomere davon oder Mischungen davon und/oder Zucker oder Zuckeralkohole, gegebenenfalls in Kombination mit oberflächenaktiven Agentien und/oder reduzierenden Agentien, ausgenommen Glycin oder Arginin.

2. Stabile Zubereitungsform nach Anspruch 1, wobei es sich bei der Transglutaminase um Faktor XIII (F XIII), biologisch aktive Fragmente, Derivate oder Muteine davon handelt.

3. Stabile Zubereitungsform nach Anspruch 2, wobei es sich um F XIII aus Plasma, Plazenta, Thrombozyten, Makrophagen/Monozyten oder um rF XIII oder um biologisch aktive Fragmente, Derivate oder Muteine davon handelt.

4. Stabile Zubereitungsform nach einem der Ansprüche 1 bis 3, wobei es sich bei den Aminosäuren um His, Glu, Met, Thr, Lys, Ala, Ile, Cys, deren Salze, Derivate, Homologe, Dimere oder Oligomere davon, oder Mischungen davon handelt.

5. Stabile Zubereitungsform nach einem der Ansprüche 1 bis 4, wobei es sich bei dem Zucker oder Zuckeralkohol um Saccharose, Maltose, Trehalose, Lactose, Sorbit oder Mannit, deren Derivate, Homologe oder Mischungen handelt.

6. Stabile Zubereitungsform nach Anspruch 5, wobei die Zucker oder Zuckeralkohole in Kombination mit der Aminosäure His, Glu, Ile und/oder Ala vorliegen.

7. Stabile Zubereitungsform nach einem der Ansprüche 1 bis 6, wobei es sich bei dem oberflächenaktiven Agens um Tween 80, Tween 20, PEG, Cetylalkohol, PVP, PVA, Lanolinalkohol oder Sorbitanmonooleat handelt.

8. Stabile Zubereitungsform nach einem der Ansprüche 1 bis 7, wobei es sich bei dem reduzierenden Agens um Cys, N-Acetyl-Cys, Thioglycerin, Natriumsulfid oder Glutathion oder Mischungen davon handelt, gegebenenfalls in Anwesenheit eines Komplexbildners.

9. Stabile Zubereitungsform nach einem der Ansprüche 1 bis 8, die als Zusätze außer einer Aminosäure(n) einen Zucker oder Zuckeralkohol und eine oberflächenaktive Substanz enthält.

10. Stabile Zubereitungsform nach Anspruch 9, die als Zusätze His/Tween 80/Saccharose, His/Tween 20/Saccharose, His/PEG/Saccharose oder His/Ile/PEG/Saccharose enthält.

11. Stabile Zubereitungsform nach einem der Ansprüche 1 bis 8, die als Zusätze (eine) Aminosäure(n) und/oder einen Zucker bzw. Zuckeralkohol, eine oberflächenaktive Substanz und gegebenenfalls ein reduzierendes Agens enthalten.

12. Stabile Zubereitungsform nach Anspruch 11, die als Zusätze (eine) Aminosäure(n)/Cys/PEG/Saccharose, (eine)Aminosäure(n)/N-Acetyl-Cys/PEG/Saccharose, (eine) Aminosäure(n) Thioglycerin/PEG/Saccharose oder Zucker/Reduktionsmittel/PEG enthält, gegebenenfalls in Anwesenheit eines Komplexbildners.

13. Stabile Zubereitungsform nach einem der Ansprüche 1 bis 12, wobei die Konzentration der Transglutaminase im Bereich von 0,003 bis 50 mg/ml liegt.

14. Stabile Zubereitungsform nach einem der Ansprüche 1 bis 13, wobei die Konzentration der Aminosäuren, deren Salze, Derivate oder Homologe im Bereich von 0,01 bis 10 % (Gew./V.), vorzugsweise von 0,1 bis 3% (Gew./V.) liegt.

15. Stabile Zubereitungsform nach einem der Ansprüche 1 bis 14, wobei die Konzentration des Zuckers oder Zuckeralkohols zwischen 0,1 und 20% (Gew./V.), vorzugsweise zwischen 0,2 und 10 %(Gew./V.) liegt.

16. Stabile Zubereitungsform nach einem der Ansprüche 1 bis 15, wobei die Konzentration des oberflächenaktiven Agens zwischen 0,00001 und 5% (Gew./V.), vorzugsweise zwischen 0,0002 bis 0,1 % (Gew./v.) liegt.

17. Stabile Zubereitungsform nach einem der Ansprüche 1 bis 16, wobei die Konzentration des reduzierenden Agens zwischen 0,001 % und 2% (Gew./V.), vorzugsweise zwischen 0,005 und 0,5% (Gew./V.) liegt.

18. Stabile Zubereitungsform nach einem der Ansprüche 1 bis 17, wobei der pH-Wert in einem Bereich von 6 bis 9, vorzugsweise zwischen 7 und 8 liegt.

19. Stabile Zubereitungsform nach Anspruch 1 bis 17 enthaltend einen Boratpuffer mit einem pH-Wert im Bereit von 6 bis 9 und gegebenenfalls einen Komplexbildner.

20. Stabile Zubereitsungsform nach Anspruch 1 bis 17 enthaltend einen Trispuffer mit einem pH-Wert im Bereich von 6 bis 9 und gegebenenfalls einen Komplexbildner.

21. Verwendung eines oder mehrerer Stabilisierungszusätze nach einem der Ansprüche 1 bis 20 zur Herstellung einer stabilen Proteinflüssigpräparation.

22. Verfahren zur Herstellung einer stabilen Proteinpräparation, umfassend das Vermischen der (des) gereinigten Proteine (Proteins) als Lösung oder Präzipitat mit einer Lösung, die einen oder mehrere Zusätze nach einem der Ansprüche 1 bis 20 enthält, und Gefriertrocknung.

23. Verfahren nach Anspruch 22, wobei es sich bei dem Protein um eine Transglutaminase handelt.

24. Verwendung der nach dem Verfahren von Anspruch 23 stabilisierten Transglutaminase zur Herstellung eines Arzneimittels.

25. Verwendung der stabilen Zubereitungsform einer Transglutaminase nach einem der Ansprüche 1 bis 20 zur Herstellung eines Arzneimittels zur Behandlung von durch F XIII-Mangel charakterisierten Krankheiten.

26. Verwendung der stabilen Zubereitungsform einer Transglutaminase nach einem der Ansprüche 1 bis 20 zur Herstellung eines Arzneimittels zur Behandlung von Erkrankungen, die durch topische oder parenterale Gabe dieser Transglutaminase positiv zu beeinflussen sind.

Europäisches Patentamt

EUROPÄISCHER RECHERCHENBERICHT

Nummer der Anmeldung
EP 96 10 1959

EINSCHLÄGIGE DOKUMENTE

Kategorie	Kennzeichnung des Dokuments mit Angabe, soweit erforderlich, der maßgeblichen Teile	Betrifft Anspruch	KLASSIFIKATION DER ANMELDUNG (Int.Cl.6)
X	EP-A-0 018 561 (BEHRINGWERKE AKTIENGESELLSCHAFT)	1-6, 13-15, 18,20-26	C12N9/10 A61K38/36
Y	*Seiten 6 bis 13 und Patentansprüche*	7,11,16	

X	EP-A-0 037 078 (THE GREEN CROSS CORPORATION)	1-6, 13-18, 21-26	
Y	*das gesamte Dokument*	7,11,16	

X	WO-A-93 15234 (NOVO NORDISK A/S)	1-6,8, 13-15, 17-19, 21-26	
Y	*Seite 2, Zeilen 5 bis 14, Seite 6, Zeile 19 bis Seite 7, Zeile 16, Patentansprüche*	7,11,16	

X	WO-A-92 00767 (CENTRE REGIONAL DE TRANSFUSION SANGUINE DE LILLE)	1-5, 13-15, 21-26	RECHERCHIERTE SACHGEBIETE (Int.Cl.6) C12N A61K
Y	*Beispiel; Patentansprüche*	7,11,16	

Y	EP-A-0 637 451 (IMMUNO AKTIENGESELLSCHAFT) *das gesamte Dokument*	7,11,16	

Der vorliegende Recherchenbericht wurde für alle Patentansprüche erstellt

Recherchenort	Abschlußdatum der Recherche	Prüfer
MÜNCHEN	8.Juli 1996	Yeats, S

KATEGORIE DER GENANNTEN DOKUMENTE

X : von besonderer Bedeutung allein betrachtet
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Veröffentlicht
Mit internationalem Recherchenbericht.

(54) Title: STABLE LYOPHILIZED PHARMACEUTICAL PREPARATIONS G-CSF

(54) Bezeichnung: STABILE LYOPHILISIERTE PHARMAZEUTISCHE ZUBEREITUNGEN VON G-CSF

(57) Abstract

The invention concerns lyophilized pharmaceutical preparations of G-CSF which contain maltose, raffinose, saccharose, trehalose or aminosugar as stabilizers. In addition, the invention concerns a method of preparing such stabilized lyophilizates and the use of maltose, raffinose, saccharose, trehalose or aminosugar as stabilizers of drugs containing G-CSF.

(57) Zusammenfassung

Gegenstand der vorliegenden Erfindung sind lyophilisierte pharmazeutische Zubereitungen von G-CSF, die Maltose, Raffinose, Saccharose, Trehalose oder Aminozucker als Stabilisierungsmittel enthalten. Außerdem betrifft die Erfindung ein Verfahren zur Herstellung dieser stabilisierten Lyophilisate, sowie die Verwendung von Maltose, Raffinose, Saccharose, Trehalose oder Aminozucker als Stabilisatoren von G-CSF-haltigen Arzneimitteln.

LEDIGLICH ZUR INFORMATION

Codes zur Identifizierung von PCT-Vertragsstaaten auf den Kopfbögen der Schriften, die internationale Anmeldungen gemäss dem PCT veröffentlichen.

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Stabile lyophilisierte pharmazeutische Zubereitungen von G-CSF

Gegenstand der vorliegenden Erfindung sind lyophilisierte pharmazeutische Zubereitungen von G-CSF, die Maltose, Raffinose, Saccharose, Trehalose oder Aminozucker als Stabilisierungsmittel enthalten. Außerdem betrifft die Erfindung ein Verfahren zur Herstellung dieser stabilisierten Lyophilisate sowie die Verwendung von Maltose, Raffinose, Saccharose, Trehalose oder Aminozucker als Stabilisatoren von G-CSF-haltigen Arzneimitteln.

Verschiedene pharmazeutische Zubereitungen, die G-CSF (Granulozyten-Kolonie stimulierender Faktor) enthalten, sind bereits aus dem Stand der Technik bekannt.

In DE 37 23 781 (GB 2,193,631) wird ein G-CSF-haltiges Arzneimittel beschrieben, das zur Stabilisierung von G-CSF mindestens ein pharmazeutisches grenzflächenaktives Mittel, Saccharid, Protein oder eine hochmolekulare Verbindung enthält. Es werden dort Zubereitungen vorgeschlagen, die Humanserumalbumin als stabilisierendes Mittel enthalten. Als vorteilhaft werden insbesondere Zubereitungen genannt, die oberflächenaktive Mittel in Gewichtsanteilen enthalten, die dem 1- bis 10 000fachen der eingesetzten G-CSF-Menge entsprechen.

In EP 0 373 679 werden stabilisierte Zubereitungen von GCSF beschrieben, die sich im wesentlichen durch einen sauren pHWert der Lösung auszeichnen, wobei die Lösungen eine möglichst geringe Leitfähigkeit aufweisen sollten. Die Lösungen besitzen einen pH-Wert von 3 - 3,7, falls die Lösungen weitere pharmazeutische Hilfsstoffe, wie beispielsweise Puffer oder Mannitol, enthalten. Für den Fall, daß keine Puffersubstanzen in der Arzneiform vorhanden sind, werden pH- Bereiche von 2,75 - 4 als vorteilhaft beschrieben.

Weiterhin werden in EP 0 306 824 stabilisierte Lyophilisate von Humanprotein-Präparaten beschrieben, bei denen die Stabilisierung durch Zusatz eines Gemisches von Harnstoff, Aminosäuren und Detergenz erfolgt.

In der früheren PCT-Patentanmeldung PCT/EP92/01823 wird ein Verfahren zur Herstellung von G-CSF-haltigen, gut verträglichen Arzneimitteln für Infusions- oder Injektionszwecke beschrieben. Die flüssigen Darreichungsformen zeichnen sich insbesondere durch eine geringe Titrationsacidität und geringe Pufferkapazität aus. Die pH-Werte der beschriebenen G-CSF- haltigen Infusions- und Injektionslösungen liegen im sauren Bereich von etwa 3,8 - 4,5.

Verfahren zur Herstellung von G-CSF-haltigen flüssigen Arzneiformen, die zusätzlich Konservierungsmittel enthalten, sind bekannt aus PCT/EP92/01822. Die pH-Werte der wässrigen pharmazeutischen Lösungen liegen im sauren Bereich von 2,5 - 4,5. Die Stabilisierung von G-CSF wird dort im wesentlichen durch die Einstellung des für G-CSF günstigen sauren pH-Wertes und durch Zugabe einer Mischung von verschiedenen Aminosäuren erreicht.

Die bisher bekannten Arzneiformen für G-CSF besitzen jedoch einige Nachteile. Es wurde festgestellt, daß flüssige GCSF-Zubereitungen gegenüber Einfrieren und Auftauen in einigen Fällen empfindlich sein können. Das unkontrollierte Einfrieren und Wiederauftauen derartiger Zubereitungen kann zur Entstehung von Dimeren, Oligomeren und Aggregaten führen, ggf. können auch unlösliche Präzipitate hervorgerufen werden. Derartige Eigenschaften von Proteinarzneimitteln sind aus medizinisch-pharmazeutischer Sicht bedenklich, da ein versehentliches Einfrieren der pharmazeutischen Lösung nicht mit Sicherheit vermieden werden kann, und somit das Risiko der Applikation einer qualitativ veränderten Zubereitung besteht.

Nachteilig bei den in DE 37 23 781 beschriebenen Zubereitungen ist ferner die Tatsache, daß diese pharmazeutische Zusatz- oder Hilfsstoffe enthalten, die aus medizinischer Sicht nicht ohne weiteres als unbedenklich einzustufen sind. Polymere und Proteine besitzen im Hinblick auf ihre Eignung als pharmazeutische Zusatzstoffe aufgrund ihrer Herkunft und physikalisch-chemischen Eigenschaften ein gewisses Risikopotential. Proteine menschlichen oder tierischen Ursprungs sowie aus Zellkulturen gewonnene Proteine tragen ein potentielles Restrisiko viraler Verunreinigungen. Auch andere, analytisch schwer nachweisbare proteinartige Verunreini-

gungen können wegen ihrer antigenen Eigenschaften immunologische Reaktionen beim Menschen hervorrufen. Proteine tierischen Ursprungs können darüber hinaus generell aufgrund ihrer spezies-spezifischen Eigenschaften immunologische Reaktionen beim Menschen auslösen. Auch Langzeitreaktionen nach späterer Reapplikation derartiger Proteine sind möglich.

Der Zusatz von hochmolekularen Verbindungen (Polymere) kann ebenfalls problematisch sein. Polymere sind aufgrund ihrer großen Molekülmasse im Körper akkumulierbar und können somit, falls kein Bioabbau erfolgt, über lange Zeit im Körper verbleiben. Dies ist besonders bei subkutaner Applikation zu befürchten, da der Abtransport und die Verteilung durch den Blutstrom gegenüber intravenöser Gabe stark verlangsamt erfolgt. In Abhängigkeit von der Molmasse können Polymere auch antigene Eigenschaften aufweisen. Auch ist die Reinheit von Polymeren aufgrund der zur Herstellung verwendeten Katalysatoren oder des Vorhandenseins von Monomeren und anderen Polymerbruchstücken schwierig zu gewährleisten. Der Einsatz von Polymeren bei pharmazeutischen Darreichungsformen, insbesondere bei subkutan applizierbaren Arzneiformen ist somit, wenn möglich, zu vermeiden.

Die in DE 37 23 781 beschriebenen Tensidmengen sind aus medizinischer Sicht ebenfalls als problematisch anzusehen. Dort werden Tensidkonzentrationen als vorteilhaft beschrieben, die bezüglich der Gewichtsanteile von G-CSF 1- bis 10 000 Gewichtsanteile eines oberflächenaktiven Mittels enthalten. Betrachtet man andererseits die für den klinischen Gebrauch bevorzugten Anwendungskonzentrationen von G-CSF von 0,05 - 1,5mg/ml in den fertigen Arzneiformen, so ergeben sich entsprechend hohe Tensidkonzentrationen. Diese sind aus medizinischer Sicht zu vermeiden, da sie lokale Irritationen auslösen können.

Außerdem haben einige der bekannten Formulierungen den Nachteil, daß sie aufgrund des angewandten niedrigen pH-Wertes insbesondere bei subkutaner Anwendung zu lokalen Unverträglichkeiten beim Patienten führen. Das erhaltene Produkt kann bei empfindlichen Patienten zu Schmerzen und lokaler Gewebereizung führen,

da der im Gewebe physiologisch vorliegende Bereich von pH 7,0 - 7,5 nicht eingehalten wird.

Aus der Literatur ist ferner bekannt, daß insbesondere nichtglykosilierte Formen von G-CSF gegenüber glykosiliertem G-CSF, das aus CHO-Cellen gewonnen wird, besonders instabil sind (J. Biol. Chem. 1990, 265, 11432). Die Stabilisierung von nicht-glykosilierten Formen von G-CSF erwies sich als besonders schwierig und bedarf speziell ausgewählter Maßnahmen, um dieses Molekül in einer stabilen Arzneiform zu formulieren.

Aufgabe der vörliegenden Erfindung war es, eine Arzneiform für G-CSF zur Verfügung zu stellen, die eine ordnungsgemäße Anwendung von G-CSF als Arzneimittel ermöglicht und die oben beschriebenen Nachteile der bisher bekannten Arzneiformen nicht aufweist. Die pharmazeutische Zubereitung sollte sowohl stabil gegenüber unkontrollierten Einfrier- und Auftauvorgängen als auch stabil bei längerer Lagerung als Lyophilisat sein, physiologisch gut verträglich, einfach handhabbar und exakt dosierbar sein.

Die in DE 37 23 781 beschriebenen Beispiele zeigen, daß stabile Lyophilisate erhalten werden können, wenn Humanserumalbumin als Hilfsstoff eingesetzt wird. Der Zusatz von Zuckeralkoholen allein führt zu weniger stabilen Formulierungen. Es ist deshalb im Sinne einer Verbesserung des Standes der Technik wünschenswert, Formulierungen zu finden, die kein Humanserumalbumin (HSA) oder andere Proteine oder Polymere enthalten und dennoch gute Stabilität auch bei erhöhter Temperatur aufweisen. Der Verzicht auf Humanserumalbumin und Polymere vermindert das medizinische Risiko von Nebenwirkungen, wie sie beispielsweise für HSA beschrieben sind.

Überraschenderweise wurde gefunden, daß man im Sinne der vorliegenden Erfindung stabile pharmazeutische Arzneiformen herstellen kann, wenn man als Zusatzstoffe Maltose, Raffinose, Saccharose, Trehalose oder Aminozucker einsetzt.

Feste Zubereitungen, die Maltose, Raffinose, Saccharose, Trehalose oder Aminozucker als Hilfsstoffe enthalten, können ohne nennenswerten Qualitätsverlust des Proteins eingefroren oder auch bei erhöhten Temperaturen (bis 40 °C) gelagert werden. Die pharmazeutische Qualität des Wirkstoffes wird hierdurch nicht negativ beeinflußt. Die erfindungsgemäßen Zubereitungen werden vorzugsweise als Lyophilisate in den Handel gebracht. Die nach Redissolution hergestellten wässrigen Zubereitungen sind sehr gut verträglich, und stellen hinsichtlich der Proteinstabilität qualitativ hochwertige Zubereitungen dar. Sie haben außerdem den Vorteil, daß durch den Zusatz von Maltose, Raffinose, Saccharose, Trehalose oder Aminozuckern als Hilfsstoffe Lösungen mit einem vorteilhaften pH-Wert von 4 - 5 oder 7 - 8 hergestellt werden können, während die aus dem Stand der Technik bekannten Lösungen aus Stabilitätsgründen des Proteins hauptsächlich Lösungen mit einem pH- Wert von 2,5 - 3,5 erforderlich machten.

Die erfindungsgemäßen Zubereitungen besitzen außerdem den Vorteil, daß sie im wesentlichen frei von proteinartigen oder polymeren Hilfsstoffen sind, deren Verwendung aus medizinischer Sicht nicht unproblematisch sein kann. Aufgrund der Tatsache, daß nunmehr durch Auflösen von Lyophilisaten erhaltene, flüssige G-CSF-haltige Arzneiformen mit einem pH-Wert von etwa 4 - 5 oder 7 - 8, vorzugsweise mit einem pH-Wert in der Nähe des pH-Wertes des Blutes (pH 7,2 - 7,4), hergestellt werden können, besitzen sie ferner den Vorteil, gut verträglich und weitgehend schmerzfrei applizierbar zu sein. Dies ist vor allem bei subkutaner Gabe wesentlich, da hier leichter Unverträglichkeiten entstehen als bei intravenöser Gabe. Die erfindungsgemäßen Zubereitungen lassen sich auch in den klinisch besonders bevorzugten Konzentrationsbereichen von 0,05 - 1,5 mg/ml herstellen, so daß Injektionsvolumina von ≤ 1,0 ml eingehalten werden können. Kleine Injektionsvolumen sind bei subkutaner Applikation besonders vorteilhaft, da sie nur geringe mechanische Reize im Unterhautgewebe hervorrufen.

Vorteilhaft ist ferner, daß aufgrund der gewählten Hilfsstoffe die bisher benötigten relativ hohen Tensidmengen in den flüssigen Arzneiformen nicht mehr erforderlich sind. Vielmehr sind niedrige Tensidmengen von 0,5 mg/ml oder weniger, vorzugs-

weise von 0,01 - 0,1 mg/ml, ausreichend zur Stabilisierung von G-CSF. Vorteilhaft können Tensidkonzentrationen (mg/ml) verwendet werden, die kleiner als oder maximal gleich der eingesetzten Proteinmenge an G-CSF pro Volumeneinheit (mg/ml) sind. Dies ist vor allem bei solchen flüssigen Arzneiformen von Vorteil, die für die subkutane Anwendung von GCSF bestimmt sind. Außerdem werden durch die erfindungsgemäßen Maßnahmen insbesondere die labilen, unglykosilierten G-CSF-Moleküle für pharmazeutische Zubereitungen ausreichend stabilisiert.

Der Hilfsstoff Maltose (Malzzucker, Maltobiose, 4-O-alpha-D-Glucopyranosyl-D-glucose) wird in einer Menge der 0,01 - 10 000fachen Menge des Wirkstoffes G-CSF eingesetzt. Das Gleiche gilt für die Hilfsstoffe Raffinose, Saccharose und Trehalose. Die Konzentration dieser Hilfsstoffe in der flüssigen Arzneiform beträgt 0,1 - 200 mg/ml, vorzugsweise 10 - 60 mg/ml. Anstatt von Maltose können auch die stereoisomeren Disaccharide Cellobiose, Gentiobiose oder Isomaltose eingesetzt werden. Als Aminozucker werden generell solche Monosaccharide bezeichnet, die anstelle einer Hydroxygruppe eine Amino- oder eine acylierte Aminogruppe besitzen. Beispiele hierfür sind Glucosamin, Galactosamin, Neuraminsäure.

In einer besonderen Ausführungsform werden pharmazeutische Zubereitungen zur Verfügung gestellt, die neben Maltose, Raffinose, Saccharose oder Trehalose ferner Aminosäuren enthalten. Insbesondere kommen als Aminosäuren basische Aminosäuren in Frage, wie beispielsweise Arginin, Lysin, Ornithin, u.a., saure Aminosäuren, wie beispielsweise Glutaminsäure, Asparaginsäure, u.a. oder auch aromatische Aminosäuren, wie beispielsweise Phenylalanin, Tyrosin, Tryptophan, u.a. .

Aminosäuren werden in einer 0,01 - 10 000fachen Menge des Wirkstoffes G-CSF eingesetzt. Die Konzentration dieser Hilfsstoffe in der flüssigen Arzneiform beträgt 0,1 - 200 mg/ml, vorzugsweise 1 - 50 mg/ml.

Zur Herstellung der Lyophilisate werden zunächst die wässrigen pharmazeutischen Lösungen hergestellt, die den Wirkstoff und andere pharmazeutische übliche Hilfs-

stoffe enthalten. Als pharmazeutische Hilfsstoffe kommen insbesondere Aminosäuren, wie z. B. Arginin, Lysin, Ornithin, Phenylalanin oder Tyrosin in Frage. Außerdem kann die wässrige Zubereitung übliche Puffersubstanzen, wie z. B. Essigsäure, Salzsäure, Zitronensäure, Milchsäure, Weinsäure, Maleinsäure und Phosphorsäure oder deren physiologisch verträglichen Salze enthalten. Bei der Herstellung der Hilfsstofflösung können diese Puffersubstanzen entweder in Form der entsprechenden freien Säure oder in Form der Alkali-, Erdalkali- oder Ammoniumsalze vorgegeben werden. Außerdem kann die Lösung weitere pharmazeutisch übliche Hilfsstoffe bereits enthalten.

Die Reihenfolge der Zugabe der verschiedenen Hilfsstoffe oder des Wirkstoffes ist weitgehend unabhängig hinsichtlich des Herstellungsverfahrens und liegt im Ermessen des Fachmannes. Der gewünschte pH-Wert der Lösung wird durch Zugabe von Basen, wie beispielsweise von Alkalihydroxiden, Erdalkalihydroxiden oder Ammoniumhydroxid eingestellt. Vorzugsweise wird hierzu Natriumhydroxid verwendet. Die Einstellung des gewünschten pH-Wertes ist prinzipiell durch Zugabe von basischen Lösungen möglich. In diesem Sinne kommen allgemein Salze von starken Basen mit schwachen Säuren in Frage, wie z. B. Natriumacetat, Natriumcitrat, Di-Natrium- bzw. Di-Kaliumhydrogenphosphat oder Natriumcarbonat. Für den Fall, daß die pharmazeutische Hilfsstofflösung einen basischen pHWert aufweist, erfolgt die Einstellung durch Titration mit Säure, bis der gewünschte pH-Bereich erreicht ist. Als Säuren kommen physiologisch verträgliche anorganische oder organische Säuren in Frage, wie beispielsweise Salzsäure, Phosphorsäure, Essigsäure, Zitronensäure oder allgemein übliche Lösungen von Substanzen, die einen sauren pHWert besitzen. Bevorzugte Substanzen sind in diesem Sinne Salze von starken Säuren mit schwachen Basen, wie z. B. Natriumdihydrogenphosphat oder Kaliumdihydrogenphosphat.

Die Konzentrationen der Puffersubstanzen in der fertig applizierbaren flüssigen Arzneiform betragen jeweils etwa 2 - 80mMol/l. Die Gesamtkonzentration an Puffersubstanzen sollte einen Wert von 100mMol/l nicht übersteigen. Bevorzugt beträgt die Konzentration der Puffersubstanzen 5 - 40 mMol/l.

Die mittels der genannten Hilfsstoffe erfolgte Stabilisierung von G-CSF-Molekülen bezieht sich prinzipiell auf alle durch rekombinante Verfahren hergestellte G-CSF-Moleküle und deren Varianten. Der Begriff G-CSF oder G-CSF-Variante gemäß vorliegender Erfindung beinhaltet alle natürlich vorkommenden Varianten von G-CSF, sowie davon abgeleitete durch rekombinante DNA-Technologie modifizierten GCSF-Proteine, insbesondere Fusionsproteine, die neben dem GCSF-Anteil noch andere Proteinsequenzen enthalten. Besonders bevorzugt ist in diesem Sinne G-CSF-Mutein mit einem N-terminalen Met-Rest an Position - 1, das zur Expression in prokaryontischen Zellen geeignet ist. Ebenso geeignet ist eine rekombinante Methionin-freie G-CSF-Variante, die gemäß PCT/EP91/00192 hergestellt werden kann. Unter dem Begriff "GCSF-Variante" werden solche GCSF-Moleküle verstanden, bei denen eine oder mehrere Aminosäuren deletiert oder durch andere Aminosäuren ersetzt sein können, wobei die wesentlichen Eigenschaften von G-CSF weitgehend erhalten bleiben. Geeignete G-CSF-Muteine sind beispielsweise in EP0 456 200 beschrieben.

Zur Herstellung gut verträglicher parenteraler Arzneiformen ist der Zusatz von isotonisierenden Hilfsstoffen zweckmäßig, wenn nicht durch die osmotischen Eigenschaften des Wirkstoffes und der zur Stabilisierung eingesetzten Hilfsstoffe bereits Isotonie erreicht werden kann. Dazu werden vor allem nicht-ionisierte, gut verträgliche Hilfsstoffe eingesetzt.

Der Zusatz von Salzen ist zur Einstellung der Isotonie nicht vorteilhaft, da hohe Salz- oder Ionenkonzentrationen die Aggregatbildung von G-CSF fördern. Vorteilhaft werden deshalb Salze in geringer Menge zugesetzt.

Außerdem können die pharmazeutischen Zubereitungen weitere übliche Hilfs- oder Zusatzstoffe enthalten. Es können Antioxidanzien, wie beispielsweise Glutathion oder Ascorbinsäure oder ähnliche Substanzen, chaotrope Hilfsstoffe, wie beispielsweise Harnstoff, und Aminosäuren, wie beispielsweise Arginin, Lysin, Ornithin, Glutaminsäure und andere zugesetzt werden.

Im folgenden wird die Erfindung anhand von repräsentativen Ausführungsbeispielen näher beschrieben:

Die Beispiele 1 - 14 zeigen, in welcher Weise erfindungsgemäße Lyophilisate formuliert, hergestellt und hinsichtlich der Stabilität des Proteins näher untersucht werden können. Der Einfluß der neben Maltose, Raffinose, Saccharose oder Trehalose zugesetzten Hilfsstoffe sowie des pH-Wertes wird erläutert.

Vergleichende Untersuchungen zu auf der Basis von Mannit oder Glycin hergestellten Lyophilisaten zeigen, daß Maltose-, Raffinose-, Saccharose- oder Trehalose-Lyophilisate signifikant bessere Ergebnisse liefern, als die mit anderen Gerüstbildnern hergestellten Zubereitungen. Durch Einsatz der erfindungsgemäß beschriebenen und in den Beispielen erläuterten Lyophilisate läßt sich im Sinne der beschriebenen Zielsetzung eine optimale Formulierung herstellen, die einen physiologisch verträglichen pH-Wert aufweist, langfristig lagerstabil ist und dabei erhöhte Lagertemperaturen sowie mechanischen Streß ohne negative Auswirkungen auf das Protein aushält. Die Zubereitungen sind insbesondere gegen Einfrieren unempfindlich und auf die als kritisch angesehenen Hilfsstoffe, wie beispielsweise Proteine oder Polymere, kann völlig verzichtet werden. Außerdem sind nur relativ geringe Mengen an physiologisch gut verträglichen Tensiden enthalten.

In Beispiel 3 werden verschiedene Zucker oder Zuckeralkohole auf ihre stabilisierende Wirkung in G-CSF-Lyophilisaten untersucht. Maltose zeigt sich gegenüber Lactose und Mannit vorteilhaft.

In Beispiel 4 werden Lyophilisate mit Maltose und weiteren Hilfsstoffen beschrieben. Die Ergebnisse belegen klar, daß der Zusatz von Tensid die Stabilität der Zubereitung nicht wesentlich beeinflußt, jedoch die Anhaftung des Proteins an Oberflächen und damit mögliche Gehaltsverluste verhindert. Die Anwesenheit von Tensid ist somit in derartigen Rezepturen nicht aus Gründen der Stabilisierung, sondern zur Aufrechterhaltung der Nenndosierung erforderlich.

In Beispiel 5 werden verschiedene Maltose-haltige Lyophilisat-Rezepturen verglichen mit zwei ansonsten gleichartig formulierten Lyophilisaten ohne Maltose. Aus den Daten ist klar erkennbar, daß die Anwesenheit von Maltose in den untersuchten Parametern vorteilhaft im Sinne der Stabilität der Zubereitung wirkt. Der Zusatz weiterer Hilfsstoffe, wie Ascorbinsäure, Glutathion, Glutaminsäure zeigt im Rahmen der untersuchten Lagertemperaturen und Lagerzeiten keinen signifikanten Einfluß auf die Stabilität. Die in Beispiel 5 beschriebenen Zubereitungen zeichnen sich insbesondere dadurch aus, daß sie bei langfristiger Lagerung bei erhöhter Temperatur keine Veränderungen in den untersuchten Qualitätsmerkmalen aufweisen.

Aus den Beispielen ist weiterhin ersichtlich, daß in Lyophilisaten, die Maltose und Arginin enthalten, ein weiteres Puffersalz nicht unbedingt erforderlich ist, da der bei pHEinstellung durch Salzsäure, Phosphorsäure, Zitronensäure oder anderen Säuren entstehende Argininpuffer ausreichend pH-stabilisierend wirkt. Argininpuffer ist hervorragend geeignet, stabile Zubereitungen im pH-Bereich unter 5,0 und von 7,0 - 7,5 zu formulieren (siehe Beispiele 11 und 12). Beispiel 9 zeigt, daß wiederaufgelöste Lyophilisate mit pH 7,4, die Maltose und Argininpuffer enthalten, mindestens 24 Stunden haltbar sind.

In Beispiel 6 werden G-CSF-Lyophilisate beschrieben, die Aminozucker (Galactosamin, N-Methylglucosamin) enthalten. Es ist erkennbar, daß die Kombination von Maltose und Aminozucker stabilere Zubereitungen ergibt als die Kombination von Glycin mit Aminozuckern. Damit wird belegt, daß Maltose in Kombination mit physiologisch gut verträglichen Hilfsstoffen deutlich stabilere und damit bezüglich der pharmazeutischen Qualität hochwertigere Lyophilisate von G-CSF liefert als andere, in der Literatur vorgeschlagene Gerüstbildner und Stabilisatoren.

In Beispiel 7 wird gezeigt, daß G-CSF in Maltose-haltigen Lyophilisaten deutlich stabiler als in Mannit-haltigen Lyophilisaten ist. Dies wird bei relevanten Lagertemperaturen und langen Lagerzeiten entsprechend belegt.

In Beispiel 8 wird gezeigt, daß Maltose-haltige Lyophilisate bei verschiedenen pH-Werten sowie bei verschiedenen Hilfsstoffzusätzen vorteilhafte Ergebnisse gegenüber Lyophilisaten mit anderen Gerüstbildnern und Stabilisatoren (Zuckeralkoholen, Aminosäuren) erbringen.

Beispiel 10 belegt die Stabilität der erfindungsgemäßen Lyophilisate mit Maltose, Raffinose, Saccharose oder Trehalose nach 13-wöchiger Lagerung bei 40 °C.

Beispiel 11 zeigt, daß die erfindungsgemäßen Lyophilisate auch mit höheren G-CSF-Konzentrationen stabil sind, und in Beispiel 12 wird die Langzeitstabilität der erfindungsgemäßen Rezepturen selbst bei höheren Temperaturen belegt.

Beispiel 1:

Untersuchungsmethoden zur Stabilitätsbestimmung

Die lyophilisierten Zubereitungen wurden unter Lichtausschluß bei definierten Lagertemperaturen gelagert und danach mit reversed phase HPLC (RP-HPLC), Gelchromatographie oder size exclusion Chromatographie (SEC HPLC), Western Blot auf Proteinreinheit sowie das Auftreten von Aggregaten und Dimeren hin untersucht. Außerdem wurde der Proteingehalt durch OD 280-Photometrie, die Biologische Aktivität durch Bioassay (NFS 60-Zelltest), sowie Aggregation und Präzipitation durch Trübungsmessung untersucht. Die angewandten Methoden lassen sich wie folgt beschreiben:

1.1 Reversed phase HPLC

Die RP-HPLC erfolgte unter Verwendung einer Nucleosil C18- Säule (Fa. Knauer). Die mobile Phase bestand aus 0,12 % (v/v) Trifluoressigsäure (TFA)/Wasser (A) und 0,1 % (v/v) TFA/Ace- tonitril (B). Die Chromatographie

wurde mit einer Flußrate von 0,5 ml/min unter Verwendung eines linearen Gradienten von A nach B durchgeführt.

Die Injektionsmenge betrug je nach Rezeptur 3 - 6 µg G-CSF. Die Auswertung erfolgte über die Peakfläche unter Verwendung eines externen Standards bei einer Wellenlänge von 214nm.

1.2 Size Exclusion Chromatographie (SEC)

Für die SE-Chromatographie wurde eine TSK G 2000 SW-Trennsäule (7,5 x 300 mm) verwendet. Die Trennungen erfolgten isokratisch bei Raumtemperatur und einer Flußrate von 0,6ml/min in einem Phosphatpuffer (22,2 mM Na_2HP04; 107,7mM KH_2PO_4; pH 6,2). Die Injektionsmenge betrug 3 - 6 µg G-CSF. Die Auswertung erfolgte über die Peakfläche unter verwendung eines externen Standards bei einer Detektionswellenlänge von 214nm.

1.3 SDS-Page/Western Blot

3 µg rhG-CSF werden unter nichtreduzierenden Bedingungen auf ein 12prozentiges Polyacrylamid-SDS-Gel gegeben und der Gelelektrophorese unterzogen. Anschließend werden die nach ihrem Molekulargewicht getrennten G-CSF-Monomere, -Dimere oder -Aggregate durch Elektroblotting auf Nitrocellulose transferiert. Durch Inkubation mit einem spezifischen polyklonalen biotinylierten Anti-G-CSF-Antikörper (PAK < GCSF >IgG) werden die Proteinbanden identifiziert und mittels der Phosphatase-Technik unter Benutzung von Streptavidin-alkalischem Phosphatasekonjugat (SA-AP-Konjugat), 5-Brom-4-chlor-3-indolylphosphat (BCIP) und Nitro Blue Tetrazolin (NBT) nachgewiesen. Die Bestimmung der prozentualen Monomeren-, Dimeren- bzw. Aggregatanteile erfolgt durch laserdensitometrische Auswertung mit Hilfe einer rhG-CSF-Standardreihe.

1.4 NFS-60 Bioassay (biologische Wirksamkeit)

Die in vitro Aktivitätsbestimmung von G-CSF basiert auf der Messung von Zellzahlen in einer durch G-CSF stimulierten Zellkultur von NFS-60 Zellen.

Unter geeigneten Bedingungen kann die Dehydrogenase-Aktivität der Zellen mit der Konzentration an G-CSF im Medium korreliert werden. Es werden geeignete Verdünnungen der G-CSF Pufferlösung hergestellt, um einen einfach meßbaren Anstieg in der Dehydrogenase-Aktivität zu erhalten.

Die Messung der Aktivität erfolgt dann photometrisch bei 570 und 690 nm, gemessen wird die Reduktion des Tetrazolinium-Salzes MTT (gelb) zu Formazan (blau).

Die in vitro Aktivität von G-CSF wird berechnet, indem die Daten der Probe gegen Standard nach der Methode der parallelen Linie verglichen werden. Die Auswertung erfolgt gemäß den Anforderungen der Ph. Eur. (VIII, 13).

1.5 Streulichtmessung, Trübungsbestimmung

Die Messung erfolgt direkt an der unverdünnten Produktlösung in Glasküvetten (Durchmesser 2 cm). Das von der Flüssigkeit diffus abgelenkte Streulicht wird unter einem Winkel von 90 °C gemessen. Gemessen wird im Vergleich zu Formazin-Standard-Suspensionen nach DIN 38404C2, die Angabe der Werte erfolgt in TE/F. Die Messung erfolgt an einem geeigneten Trübungsphotometer, z. B. LTP 5 (Fa. Dr. Lange, Düsseldorf).

1.6 Photometrie OD 280 (Proteingehalt)

Das G-CSF-UV-Spektrum hat ein Absorptionsmaximum bei 280 nm, das auf Seitenkettenchromophore wie Tryptophan-, Tyrosin- und Phenylalaninreste zurückzuführen ist. Die Messung erfolgt im Vergleich zu Placebo-Lösungen mittels:

- UV-Spectrophotometer
  (z. B. Uvikon 810 P oder 941, Kontron Instruments)
- Semi-micro Quartz Küvetten, 500 µl, Schichtdicke: 1 cm
  (z. B. Hellma, Suprasil, Cat. No. 104.002B-QS)

Beispiel 2:

Wässrige Lösungen von 0,1 mg/1 ml Poloxamer 118 sowie 50mg/ml der nachfolgend genannten Zucker bzw. Zuckeralkohole Mannit (Rezeptur 1), Lactose (Rezeptur 2) und Maltose (Rezeptur 3) wurden mit G-CSF in einer Konzentration von 70 µg/ml versetzt. Die Lösungen wurden nach Filtration durch einen sterilisierten 0,2 µm-Membranfilter in sterile Injektionsflaschen aus Glas der hydrolytischen Klasse I abgefüllt. Nach der Lyophilisation wurde mit sterilem Stickstoff belüftet und die zunächst lose aufgesetzten Stopfen wurden zum Verschließen der Lyophilisate unter aseptischen Bedingungen eingedrückt. Die Lyophilisate wurden verbördelt und unter Lichtausschluß 6 und 13 Wochen bei verschiedenen Temperaturen gelagert. Danach wurde mit den nachfolgend beschriebenen Methoden die Stabilität der Zubereitung untersucht.

Tabelle 1a: Lagerung bei 20 °C

	Lagerung 6 Wochen bei 20 °C			Lagerung 13 Wochen bei 20 °C		
	I	II	III	I	II	III
Rez. 1 Mannit	> 99 %	91 %	36 %	86 %	47 %	27 %
Rez. 2 Lactose	> 99 %	> 99 %	18 %	> 99 %	> 99 %	12 %
Rez. 3 Maltose	> 99 %	> 99 %	6 %	> 99 %	> 99 %	7,8 %

I Reinheit unverändertes Protein in RP HPLC
II Reinheit unverändertes Protein in SEC HPLC
III Dimeren/Aggregate in Western Blot

Tabelle 1b: Lagerung bei 40 °C

	Lagerung 6 Wochen bei 40 °C			Lagerung 13 Wochen bei 40 °C		
	I	II	III	I	II	III
Rez. 1 Mannit	81 %	70 %	50 %	69 %	25 %	70 %
Rez. 2 Lactose	> 99 %	> 99 %	37 %	> 99 %	> 99 %	nicht auswertbar/ aggregiert
Rez. 3 Maltose	> 99 %	> 99 %	6,4 %	> 99 %	> 99 %	12 %

I Reinheit unverändertes Protein in RP HPLC
II Reinheit unverändertes Protein in SEC HPLC
III Dimeren/Aggregate in Western Blot

Beispiel 3:

Es wurden Lyophilisate von GCSF hergestellt. Dazu wurden die in nachfolgender Tabelle genannten Hilfsstoffe in Wasser für Injektionszwecke gelöst, danach wurde G-CSF in einer Konzentration von 70 µg/ml zugefügt und ggf. wurde mit geringen Mengen des Puffersystems der pHWert genau eingestellt. Pluronic F 68 wurde als ein Vertreter eines entsprechend geeigneten Tensides verwendet. Andere Tenside verhalten sich ähnlich. Nach Sterilfiltrationen durch einen geeigneten 0,2 µm-Membranfilter wurden die Lösungen in sterile Injektionsflaschen aus Glas der hydrolytischen Klasse I abgefüllt und nach üblichen Verfahren lyophilisiert. Nach der Lyophilisation wurde mit Stickstoff belüftet und die Injektionsflaschen mit Gefriertrocknungsstopfen unter aseptischen Bedingungen verschlossen. Die Zubereitungen wurden in verbördelten Flaschen unter Lichtausschluß bei definierten Lagertemperaturen bei 6 und 12 Wochen gelagert und mit den in Beispiel 1 genannten Methoden untersucht.

Tabelle 2: Maltose-haltige Rezepturen von G-CSF bei pH 3,6

	Rezeptur 4	Rezeptur 5
G-CSF	70 µg	70 µg
Maltose	35 mg	35 mg
L-Phenylalanin	10 mg	10 mg
Ascorbinsäure	5 mg	5 mg
Glutathion	10 mg	10 mg
L-Glutaminsäure	5 mg	5 mg
L-Arginin	10 mg	10 mg
Puffer (pH)	ad pH 3,6	ad pH 3,6
Pluronic F 68	-	0,1 mg
Wasser für Injektionszwecke	ad 1 ml	ad 1 ml

Tabelle 3: Lagerung bei 20 °C

Rp.	Lager-temp.	nach 6 Wochen Aggr. in %	nach 12 Woche Aggr. in %	nach 6 Wochen SEC % G-CSF	nach 6 Wochen RP % G-CSF	nach 12 Wochen SEC % G-CSF	nach 12 Wochen HPLC % G-CS
4	+ 20 °C	0,0	2,6	61	63	64	69
5	+ 20 °C	0,0	0,5	> 99	> 99	> 99	> 99

Beispiel 4:

G-CSF-Lyophilisate mit 500 µg/ml G-CSF (Rezepturen 6 - 10) wurden wie folgt hergestellt. Die in nachfolgender Tabelle genannten Hilfsstoffe wurden in Wasser für Injektionszwecke gelöst, GCSF wurde zugegeben und, falls nötig, wurde der pHWert mit geringen Mengen Salzsäure oder oder Di-Natrium-hydrogenphosphat einjustiert. Jeweils 1 ml der zuvor durch ein 0,2 µm-Membranfilter sterilfiltrierten Lösungen wurde in Injektionsflaschen aus Glas der hydrolytischen Klasse I abgefüllt und nach üblichen Verfahren gefriergetrocknet. Nach der Lyophilisation wurde mit Stickstoff belüftet und die Lyophilisate wurden mit Gefriertrocknungsstopfen unter aseptischen Bedingungen verschlossen. Die verbördelten Lyophilisate wurden unter Lichtausschluß bei definierten Temperaturen gelagert und mit den in Beispiel 1 genannten Methoden untersucht.

Tabelle 4: Zusammensetzungen der Rezepturen 6 - 10

	Rez. 6	Rez. 7	Rez. 8	Rez. 9	Rez. 10
G-CSF	0,5 µg	0,5 µg	0,5 µ	0,5 µg	0,5 µg
Maltose	35 mg	35 mg	35 mg	-	-
L-Phenylalanin	10 mg	10 mg	10 mg	10 mg	10 mg
Ascorbinsäure	5 mg	-	-	5 mg	-

	Rez. 6	Rez. 7	Rez. 8	Rez. 9	Rez. 10
Glutathion	10 mg	-	-	10 mg	-
L-Glutaminsäure	5 mg	-	-	5 mg	-
L-Arginin	10 mg	10 mg	10 mg	10 mg	10 mg
Puffer (pH)	ad 4,5	ad 4,5	ad 6,5	ad 4,5	ad 6,5
Pluronic F68	0,1 mg	0,1 mg	0,1 mg	0,1 mg	0,1 mg
Wasser für Injektionszw.	ad 1 ml	ad 1 ml	ad 1 ml	ad 1 ml	ad 1 ml

Tabelle 5: Analysenergebnisse

Rez.	Lager-temp.	Western Blot		nach 6 Wochen			nach 13 Wochen		
		6 Wo. % Aggr.	12 Wo. % Aggr.	SEC % G-CSF	% Aggr.	RP % G-CSF	SEC % G-CSF	% Aggr.	RP % G-CSF
6	+ 8 °C	< 1	1,0	> 99 %	0,9	> 99 %	> 99 %	0,7	99 %
	+ 40 °C	< 1	1,7	> 99 %	0,6	> 99 %	> 99 %	0,6	98 %
7	+ 8 °C	< 1	1,1	> 99 %	1,6	> 99 %	> 99 %	1,1	99 %
	+ 40 °C	< 1	2,3	> 99 %	1,9	> 99 %	> 99 %	1,1	99 %
8	+ 8 °C	< 1	-				> 98 %	1,5	> 99 %
	+ 40 °C	< 1	-				> 98 %	1,4	> 99 %
9	+ 8 °C	3,8	0,3	95 %	5,8	> 99 %	95 %	1,5	98 %
	+ 40 °C	7,9	2,5	95 %	6,4	93 %	86 %	0,8	94 %
10	+ 8 °C	-	5,2				96 %	1,0	95 %
	+ 40 °C	-	10,3				89 %	2,6	89 %

Beispiel 5:

Die in der nachfolgenden Tabelle angegebenen Formulierungen (Rezepturen 11 - 14) wurden wie folgt hergestellt: Die Hilfsstoffe wurden in Wasser für Injektionszwecke gelöst, danach G- CSF in der angegebenen Konzentration zugefügt. Falls notwendig, wurde der pH mit Hilfe der Komponenten des Phosphatpuffers genau einjustiert. Die Lösungen wurden sodann durch einen sterilisierten Membranfilter der Porenweite 0,2 µm filtriert und unter aseptischen Bedingungen in Injektionsflaschen der hydrolytischen Klasse I abgefüllt und lyophilisiert. Nach der Lyophilisation wurde mit Stickstoff belüftet, die Lyophilisate wurden mit Gefriertrocknungsgummistopfen unter aseptischen Bedingungen verschlossen und verbördelt. Die Lyophilisate wurden unter Lichtausschluß bei definierten Lagertemperaturen belastet. Nach 6 und 13 Wochen wurden Untersuchungen gemäß der in Beispiel 1 angegebenen Methoden durchgeführt.

Tabelle 6: Aminozuckerhaltige Lyophilisatzubereitungen

	Rez. 11	Rez. 12	Rez. 13	Rez. 14
G-CSF	0,5 µg	0,5 µg	0,5 µg	0,5 µg
Pluronic F68	0,1 mg	0,1 mg	0,1 mg	0,1 mg
N-methyl-glucosamin	-	10 mg	-	10 mg
Galactosamin	10 mg	-	10 mg	-
Glycin	-	-	35 mg	35 mg
Maltose	35 mg	35 mg	-	-
Phenylalanin	10 mg	-	-	-
Phosphatpuffer	ad pH 7,0	ad pH 7,0	ad pH 7,0	ad pH 7,0
Wasser für Injektionszwecke	ad 1,0 ml	ad 1,0 ml	ad 1,0 ml	ad 1,0 ml

Die nach Lagerung der o. g. Zubereitung erhaltenen Analysendaten sind in nachfolgender Ergebnistabelle zusammengefaßt.

Tabelle 7: Analysenergebnisse
ZSP = Zersetzungsprodukte

Rez.	Lagerung	6 Wochen West. Blot. % Aggr.	12 Wochen West. Blot. % Aggr.	12 Wochen RP-HPLC % G-CSF	12 Wochen % ZSP in SEC HPLC
11	+ 8 °C	3,8	2,9	> 99	1,2
	+ 40 °C	3,2	2,3	> 99	1,8
12	+ 8 °C	1,8	3,8	> 99	1,4
	+ 40 °C	1,7	4,5	> 99	0,7
13	+ 8 °C	1,1	1,4	> 99	0,9
	+ 40 °C	16,8	13,0	75	4,2
14	+ 8 °C	1,6	12,4	97,5	1,2
	+ 40 °C	7,7	26,3	84,5	3,5

Beispiel 6:

Die nachfolgend beschriebenen Rezepturen 15 und 16 wurden wie folgt hergestellt: Die angegebenen Hilfsstoffe wurden in Wasser für Injektionszwecke gelöst, G-CSF in der angegebenen Konzentration wurde zugefügt. Der pH wurde, falls notwendig, mit Anteilen der Pufferkomponenten einjustiert. Danach wurde die Lösung durch einen sterilisierten Membranfilter der Porenweite 0,2 µm filtriert und unter aseptischen Bedingungen in sterile Injektionsflaschen aus Glas der hydrolytischen Klasse I abgefüllt. Nachfolgend wurden die Injektionszubereitungen gefriergetrocknet, danach wurde mit Stickstoff belüftet und die Injektionsflaschen wurden unter aseptischen Bedingungen mit einem Gefriertrocknungsstopfen verschlossen und danach verbördelt. Die Zubereitungen wurden unter Lichtausschluß bei definierten Temperaturen gelagert und auf die nachfolgend genannten Parameter hin untersucht. Dabei wurden die in Beispiel 1 beschriebenen Untersuchungsmethoden angewandt.

Rezeptur 15

G-CSF	0,25 mg
Polysorbat 80	0,05 mg
Phenylalanin	5 mg
Maltose	17,5 mg
L-Arginin	5 mg
Puffer	ad pH 4,5
Wasser f. Injektionszw.	ad 0,5 ml

Rezeptur 16

G-CSF	0,3 mg
Polysorbat 80	0,1 mg
Mannit	50 mg
Puffer	ad pH 4,5
Wasser f. Injektionszw.	ad 1,0 ml

Tabelle 8: Untersuchungsergebnisse nach Lagerung von Rezeptur 15 und 16 über 3 und 6 Monate

	Rezeptur 15				Rezeptur 16			
	Lagerzeit 3 Monate		Lagerzeit 6 Monate		Lagerzeit 3 Monate		Lagerzeit 6 Monate	
	4-8 °C	23 °C	4-8 °C	23 °	4-8 °C	23 °C	4-8 °C	23 °
West. Blot (Dimere)	2,2	< 1 %	1,3 %	0,7	< 1 %	12 %	4,1 %	17
SEC-HPLC (Dimere)	< 1 %	< 1 %	< 1 %	< 1 %	< 1 %	2 %	< 1 %	3
RP-HPLC (G-CSF Peak)	> 99 %	> 99 %	> 98 %	> 98	> 99 %	98,2 %	> 98 %	> 98

Beispiel 7:

Die in Tabelle 9 beschriebenen Formulierungen wurden wie folgt hergestellt:

Die angegebenen Hilfsstoffe wurden in Wasser für Injektionszwecke gelöst, G-CSF wurde in der angegebenen Konzentration zugegeben, danach wurde, falls notwendig, der pHWert mit kleinen Anteilen der Pufferkomponenten einjustiert. Die Arzneistoff-lösung wurde dann durch einen sterilisierten Membranfilter der Porenweite 0,2 µm sterilfiltriert, danach unter aseptischen Bedingungen in sterilisierte Injektionsflaschen aus Glas der hydrolytischen Klasse I abgefüllt und lyophilisiert.

Nach der Lyophilisation wurde mit Stickstoff belüftet und die Flaschen wurden unter aseptischen Bedingungen mit Gefriertrocknungsstopfen verschlossen. Die Flaschen wurden verbördelt und unter Lichtausschluß und definierten Temperaturbedingungen gelagert. Nach den entsprechenden Lagerzeiten wurden mit den in Beispiel 1 beschriebenen Methoden analytische Untersuchungen durchgeführt (vgl. Tabelle 10).

Tabelle 9: G-CSF Lyophilisate mit Maltose im Vergleich zu anderen Gerüstbildern

	Rez. 17	Rez. 18	Rez. 19	Rez. 20	Rez. 21	Rez. 22	Rez. 23	Rez. 24
G-CSF	350 µg	350 µg	175 µg	175 µg	175 µg	175 µg	175 µg	175 µg
Maltose			17,5 mg	17,5 mg	17,5 mg	-	-	-
Glycin						4 mg	10 mg	8,9 mg
Arginin			5 mg	-	5 mg	5 mg	-	
Phenylalanin			5 mg	5 mg	5 mg	5 mg	-	2,5 mg
Mannit	50 mg	50 mg				-	-	
Tween 80	0,1 mg	0,1 mg	0,05 mg	0,05 mg	0,05 mg	0,05 mg	0,05 mg	0,05 mg
pH	4,5	7,2	4,5	7,2	7,2	7,2	7,2	7,2
(Puffer)	HCl	Phosphat	HCl	HCl	HCl	HCl	Phosphat	Phosphat
Wasser für Injektionszwecke	ad 1,0 ml	ad 1,0 ml	ad 0,5 ml	ad 0,5 ml	ad 0,5 ml	ad 0,5 ml	ad 0,5 ml	ad 0,5 ml

Tabelle 10 sind die erhaltenen Analysendaten für die genannten Rezepturen zusammengefaßt:

Rezeptur	Lagerung	4 Wochen RP-HPLC % G-CSF	4 Wochen SEC-HPLC % G-CSF	4 Wochen Western Blot
17	8 °C	> 99	> 99	3,6 % Dimere
	30 °C	94	92	9,6 % Dimere
	40 °C			14,4 % Dimere
18	8 °C	69	60	Aggregate
	30 °C	44	36	Aggregate
	40 °C	13	12	Aggregate
19	8 °C	> 99	> 99	1,0 % Dimere
	30 °C	> 99	95, 5	0,5 % Dimere
	40 °C	> 99	97,5	0,5 % Dimere
20	8 °C	> 99	> 99	1,6 % Dimere
	30 °C	> 99	> 99	1,4 % Dimere
	40 °C	> 99	> 99	2,3 % Dimere
21	8 °C	> 99	> 99	1,5 % Dimere
	30 °C	> 99	97,5	2,1 % Dimere
	40 °C	> 99	97	2,0 % Dimere
22	8 °C	> 99	> 99	2,8 % Di/Aggregat
	30 °C	96	96	3,0 % Di/Aggregat
	40 °C			12 % Di/Aggregate
23	8 °C	> 99	> 99	6,8 % Dimere
	30 °C	91,5	92	Aggregate
	40 °C	79	74	Aggregate
24	8 °C	> 99	> 99	10,8 % Dimere
	30 °C	88	85	Aggregate
	40 °C	67	60	Aggregate

Beispiel 8:

Standzeit erfindungsgemäßer wiederaufgelöster Lyophilisate mit pH 7,4

Es wurde folgende Zusammensetzung hergestellt:

mg/ml	Rezeptur 25
G-CSF	0,35
Polysorbat 80	0,1
Maltose	50
Arginin	10
Phenylalanin	10
Salzsäure	ad pH 7,4

Die genannten Hilfsstoffe wurden in 1 ml Wasser für Injektionszwecke gelöst, G-CSF wurde zugefügt und der pH-Wert auf pH 7,4 eingestellt. Die Lösung wurde durch einen sterilisierten Membranfilter der Porenwerte 0,2 µm sterilfiltriert und danach in Injektionsflaschen der hydrolytischen Klasse 1 abgefüllt.

Nach dem Aufsetzen geeigneter Gefriertrocknungsstopfen wurde die Zubereitung bei einer Haupttrocknungstemperatur von - 25 °C und einer Nachtrocknungstemperatur von + 8 °C bis zu einer Restfeuchte von < 5 % gefriertrocknet. Die getrockneten Lyophilisate wurden mit Stickstoff belüftet und verschlossen.

Nach 6monatiger Lagerung bei 4 - 8 °C wurden die Lyophilisate mit 1 ml Wasser für Injektionszwecke aufgelöst und danach 24 Stunden bei Raumtemperatur stehengelassen.

Nach dieser Standzeit zeigte sich mit den in Beispiel 1 beschriebenen Untersuchungsmethoden zur biologischen Wirksamkeit (NFS-60 Test), Proteingehalt (Photometrie OD 280) und Reinheit (Western Blot), Reinheit (SEC HPLC), Reinheit

(SDS Page) und Reinheit (RP HPLC) keine Veränderung gegenüber den unmittelbar nach dem Auflösen untersuchten Proben. Auch Trübungsmessungen - selbst unter mechanischer Belastung - ergaben sehr niedrige Trübungswerte.

Damit wird deutlich, daß wiederaufgelöste Lyophilisate der erfindungsgemäßen Rezeptur mit Maltose und Argininpuffer bei pH 7,4 eine für die klinische Anwendung ausreichende Standzeit besitzen.

Beispiel 9:

Stabilität erfindungsgemäßer Lyophilisate, die Maltose, Raffinose, Saccharose oder Trehalose enthalten, nach 13-wöchiger Lagerung bei 40 °C

Gemäß Beispiel 8, Rezeptur 25, wurden drei Lyophilisate hergestellt, die 50 mg/ml Maltose oder die gleiche Gewichtsmenge von a) Raffinose oder b) Saccarose oder c) Trehalose enthielten.

Alle Lyophilisate wurden 13 Wochen bei Temperaturen von 5 °C, 25 °C, 30 °C und 40 °C eingelagert, danach aufgelöst und visuell und mit den in Beispiel 1 beschriebenen Untersuchungsmethoden SEC HPLC, RP HPLC, Western Blot und SDS Page untersucht.

In allen Fällen ergaben sich klare farblose Lösungen. In der SEC HPLC betrug die Größe der Produktpeaks > 98 % und die der Dimeren/Aggregate < 1 %. In der RP HPLC erreichte der Produktpeak 100 %, Nebenpeaks waren nicht nachweisbar, der Hauptpeak entsprach dem Arbeitsstandard. Im SDS-Page waren keine Abbauprodukte, Dimere oder Aggregate nachweisbar.

Tabelle: nach 13 Wochen Lagerzeit

Temp.	SEC HPLC % G-CSF	RP HPLC % G-CSF	Western Blot % Aggregate	SDS-Page % Nebenbande
5 °C	> 98 %	100 %	n.n.	< 1
20 °C	> 98 %	100 %	n.n.	< 1
30 °C	> 98 %	100 %	n.n.	< 1
40 °C	> 98 %	100 %	n.n.	< 1

Beispiel 10:

Stabilität erfindungsgemäßer Maltose-Lyophilisate mit Argininphosphat- und Argininchloridpuffern mit pH 4,5 und pH 7,2 nach 13-wöchiger Lagerung bei 30 °C

Gemäß der in Beispiel 8 beschriebenen Herstellung wurden die folgenden Rezepturen, die sich lediglich durch Puffer und pH-Wert unterscheiden, hergestellt:

	Rezeptur 26	Rezeptur 27	Rezeptur 28	Rezeptur 29
G-CSF	0,35 mg	0,35 mg	0,35 mg	0,35 mg
Polysorbat 80	0,1 mg	0,1 mg	0,1 mg	0,1 mg
Phenylalanin	10 mg	10 mg	10 mg	10 mg
Arginin	10 mg	10 mg	10 mg	10 mg
Maltose	47,5 mg	47,5 mg	47,5 mg	47,5 mg
Phosphorsäure	ad pH 4,5	ad pH 7,2		
Salzsäure			ad pH 4,5	ad pH 7,2

Die Zubereitungen wurden bei Temperaturen von 4 bis 8 °C, 20 - 25 °C sowie 30 °C eingelagert, nach 13 Wochen in 1 ml Wasser für Injektionszwecke aufgelöst und mit den in Beispiel 1 beschriebenen Untersuchungsmethoden RP HPLC, SEC HPLC (Reinheit) und Western Blot (Abbau, Dimerisation und Aggregatbildung) untersucht. Die Ergebnisse sind in Tabelle 11 dargestellt und zeigen, daß die erfindungsgemäßen Lyophilisate mit pH 4,5 und 7,2 auch nach 13-wöchiger Lagerung bei 30 °C stabil sind.

Tabelle 11: Ergebnisse nach 13 Wochen 30 °C

	RP-HPLC	SEC HPLC	Western Blot		
	% Nebenpeaks		Abbau	Dimere	Aggregat
Rezeptur 26	< 1	< 1	n.n.	n.n.	n.n.
Rezeptur 27	< 1	< 1	n.n.	n.n.	n.n.
Rezeptur 28	< 1	< 1	n.n.	< 1 %	n.n.
Rezeptur 29	< 1	< 1	n.n.	< 1 %	n.n.

n.n. = nicht nachweisbar

Beispiel 11:

Stabilität erfindungsgemäßer Maltose-Lyophilisate mit Argininphosphat- und Argininchloridpuffer mit pH 7,4 nach 4- und 13-wöchiger Lagerung bei 40 °C

Es wurden in gleicher Weise wie in Beispiel 8 Rezepturen hergestellt und deren pH-Wert einmal mit Salzsäure und einmal mit Phosphorsäure auf 7,4 eingestellt:

	Rezeptur 30	Rezeptur 31
G-CSF	0,35 mg	0,35 mg
Polysorbat 80	0,1 mg	0,1 mg
Phenylalanin	10 mg	10 mg
Arginin	10 mg	10 mg
Maltose	47,5 mg	47,5 mg
Phosphorsäure	pH 7,4	
Salzsäure		pH 7,4

Diese zwei Zubereitungen wurden bei Temperaturen von 4 - 8 °C sowie 40 °C 4 und 13 Wochen eingelagert. Die Untersuchungsergebnisse (Western Blot, SDS-Page) nach 13 Wochen Lagerung sind in nachfolgender Tabelle 12 dargestellt. Die Ergebnisse nach 4-wöchiger Lagerung sind identisch.

Die Ergebnisse belegen, daß die erfindungsgemäßen Maltose-Lyophilisate mit pH 7,4 auch nach 13-wöchiger Lagerung bei 40 °C stabil sind.

Tabelle 12: Ergebnisse nach 13 Wochen 40 °C

	Lager temp.	Western Blot, nicht reduz.		SDS-Page, reduz.			Rest-feuchte %
		Aggr. < 1 %	Dimere < 2 %	Produkt-Bd.	Abbau-prod.	Zusatz-bd.	
Rezeptur 30	5 °C	n.n.	n.n.	< 98 %	< 1 %	n.n.	1,4
	40 °C	n.n.	n.n.	< 98 %	< 1 %	n.n.	2,0
Rezeptur 31	5 °C	n.n.	n.n.	< 98 %	< 1 %	n.n.	1,9
	40 °C	n.n.	n.n.	< 98 %	< 1 %	n.n.	2,1

Beispiel 12:

Stabilität erfindungsgemäßer Lyophilisate mit pH 7,4 und G-CSF-Konzentrationen von 0,5 und 1,0 mg/ml nach 13-wöchiger Lagerung bei 40 °C

Gemäß der in Beispiel 8 beschriebenen Herstellung wurden die folgenden Rezepturen, die sich im G-CSF-Gehalt unterscheiden, hergestellt:

mg/ml	Rezeptur 32	Rezeptur 33
G-CSF	0,5 mg	1,0 mg
Polysorbat 80	0,1 mg	0,1 mg
Phenylalanin	10 mg	10 mg
Arginin	10 mg	10 mg
Maltose	47,5 mg	47,5 mg
Phosphorsäure	pH 7,4	pH 7,4

Die Zubereitungen wurden bei - 20 °C, 4 - 8 °C, 20 °C - 25 °C, 30 °C und 40 °C 4 Wochen und 13 Wochen gelagert, danach in 1 ml Wasser für Injektionszwecke aufgelöst und mit den in Beispiel 1 beschriebenen Untersuchungsmethoden SEC HPLC, RP HPLC, Western Blot und SDS Page untersucht (Untersuchungsergebnisse siehe Tabelle 13).

Die Ergebnisse zeigen, daß die Lyophilisate der erfindungsgemäßen Rezepturen auch bei höherer Proteinkonzentration bis zu 1 mg/ml nach 13-wöchiger Lagerung bei 40 °C stabil sind.

Beispiel 14 :

Langzeitstabilität über 9 Monate

Es wurden Lyophilisate gemäß der Rezeptur 31 nach Beispiel 11 hergestellt und die Zubereitungen bei Temperaturen von - 20 °C, 5 °C, 25 °C, 30 °C und 40 °C über 9 Monate gelagert und nach 3, 6 und 9 Monaten mit allen in Beispiel 1 beschriebenen Untersuchungsmethoden untersucht.

In allen geprüften Parametern war im Laufe der Lagerzeit keine Veränderung nachweisbar. Die Zubereitung erwies sich am Ende der Lagerzeiten bei allen Temperaturen als biologisch voll wirksam, wies den vollen Proteingehalt auf und zeigte in allen Reinheitsbestimmungen Banden bzw. Peaks, die weit unter 1 % des intakten G-CSF-Moleküls lagen.

Die Ergebnisse belegen, daß die erfindungsgemäßen Lyophilisate langfristig auch bei höheren Temperaturen stabil sind und somit die im Stand der Technik beschriebenen Stabilitäten bei weitem übertreffen.

Tabelle 14: Lagerung bei 30 °C

	3 Monate	6 Monate	9 Monate
NFS 60 Test 80 - 125 %	entspricht	entspricht	entspricht
OD 280	358 mg	360 mg	352 mg
SDS Page Nebenbande	< 1 %	< 1 %	< 1 %
Western Blot % Aggr. % Dimere	n.n. < 1 %	n.n. < 1 %	n.n. < 1 %

	3 Monate	6 Monate	9 Monate
RP HPLC Produktpeak	> 99 %	> 99 %	> 99 %
SEC HPLC Produktpeak Nebenpeaks	> 98 % n.n.	> 98 % n.n.	> 98 % n.n.
Trübungsmessung TE/F	0,5	0,5	0,5

Tabelle 13:

Prüfparameter	Rezeptur 32						Rezeptur 33					
	0 Wo [KS]	13 Wo [-20°C]	13 Wo [KS]	13 Wo [RT]	13 Wo [30 °C]	13 Wo [40 °C]	0 Wo [KS]	13 Wo [-20°C]	13 Wo [KS]	13 Wo [RT]	13 Wo [30 °C]	13 Wo [40 °C]
Visuelle Prüfung							-	weiß	weiß	weiß	weiß	weiß
- Aussehen Lyophilisat	-	weiß	weiß	weiß	weiß	weiß	-	klar,	klar,	klar,	klar,	klar,
- Klarheit, (Lösung)	-	klar,	klar,	klar,	klar,	klar,						
SEC-HPLC												
[Reinheit %]	> 98	> 98	> 98	> 98	> 98	> 98	> 98	> 98	> 98	> 98	> 98	> 98
[Dimere/Aggregate %]	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1
RP-HPLC												
[Reinheit %]	> 98	> 98	> 98	> 98	> 98	> 98	> 98	> 98	> 98	> 98	> 98	> 98
[Summe der Nebenpeaks %]	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1	< 1
Western-Blot [Dimere %]	n.n.	n.n.	n.n.	n.n.	n.n.	n.n.	n.n.	n.n.	n.n.	n.n.	n.n.	n.n.
[Aggregate %]	n.n.	n.n.	n.n.	n.n.	n.n.	n.n.	n.n.	n.n.	n.n.	n.n.	n.n.	n.n.
[Abbauprodukte %]	n.n.	n.n.	n.n.	n.n.	n.n.	n.n.	n.n.	n.n.	n.n.	n.n.	n.n.	n.n.
SDS-PAGE, silver stain [Monomer %]	-	>> 99%	>> 99%	>> 99%	>> 99%	>> 99%	-	>> 99%	>> 99%	>> 99%	>> 99%	>> 99%
[Zusatzbanden %]	-	n.n.	n.n.	n.n.	n.n.	n.n.	-	n.n.	n.n.	n.n.	n.n.	n.n.
[Abbauprodukte %]	-	<< 1%	<< 1%	<< 1%	<< 1%	<< 1%	-	<< 1%	<< 1%	<< 1%	<< 1%	<< 1%

n.n. = nicht nachweisbar

Patentansprüche

1. Lyophilisierte pharmazeutische Zubereitung von G-CSF, enthaltend Maltose, Raffinose, Saccharose, Trehalose oder Aminozucker.

2. Lyophilisierte Zubereitung gemäß Anspruch 1, enthaltend zusätzlich eine physiologisch verträgliche Menge an Tensiden, die kleiner als oder maximal gleich der eingesetzten Proteinmenge an G-CSF ist.

3. Lyophilisierte Zubereitung gemäß Anspruch 2, enthaltend 0,5 mg/ml, vorzugsweise 0,01 - 0,1 mg/ml Tenside.

4. Lyophilisierte Zubereitung gemäß einem der Ansprüche 1 bis 3, enthaltend zusätzlich eine physiologisch verträgliche Menge an Aminosäuren.

5. Lyophilisierte Zubereitung gemäß Anspruch 4, enthaltend Arginin und/oder Phenylalanin.

6. Lyophilisierte Zubereitung gemäß einem der Ansprüche 1 bis 5, enthaltend physiologisch verträgliche Hilfsstoffe ausgewählt aus der Gruppe der Antioxidantien, Komplexbildner, Puffer, Säuren, Basen oder Isotonisierungsmitttel.

7. Lyophilisierte Zubereitung gemäß einem der Ansprüche 1 - 6 enthaltend Phosphat- oder Acetatpuffer.

8. Lyophilisierte Zubereitung gemäß einem der Ansprüche 1 - 6, enthaltend Argininphosphat-, Argininchlorid- oder Argincitratpuffer, vorzugsweise mit einem pH-Wert von 7 - 8.

9. Lyophilisierte Zubereitung gemäß einem der Ansprüche 1 bis 8, dadurch gekennzeichnet, daß sie im wesentlichen frei sind von proteinartigen Hilfsstoffen oder polymeren Hilfsstoffen.

10. Wässrige pharmazeutische Zubereitung, erhältlich durch Redissolution des Lyophilisates gemäß einem der Ansprüche 1 bis 9.

11. Wässrige pharmazeutische Zubereitung gemäß Anspruch 10, dadurch gekennzeichnet, daß die Lösung einen pH-Wert von 6,5 - 8 oder 3 - 5 aufweist, vorzugsweise einen pH-Wert von 7,0 - 7,5.

12. Verfahren zur Herstellung einer lyophilisierten pharmazeutischen Zubereitung gemäß einem der Ansprüche 1 - 9, dadurch gekennzeichnet, daß man eine wässrige Zubereitung, enthaltend G-CSF als Wirkstoff und Maltose, Raffinose, Saccharose, Trehalose oder Aminozucker als Hilfsstoffe sowie ggf. weitere pharmazeutische Hilfsstoffe, herstellt und die Lösung anschließend lyophilisiert.

13. Verwendung von Maltose, Raffinose, Saccharose, Trehalose oder Aminozucker zur Herstellung einer stabilen lyophilisierten pharmazeutischen Zubereitung, enthaltend G-CSF als Wirkstoff.

INTERNATIONAL SEARCH REPORT

In. ational Application No
PCT/EP 93/03543

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 A61K37/02 A61K9/14 A61K47/18 A61K47/26

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE,A,37 23 781 (CHUGAI SEIYAKU K.K.) 21 January 1988 cited in the application see claims 1-5,10-13 see page 4, line 30 - line 54 see page 4, line 57 - line 65 see page 5, line 16 - line 19 see page 5, line 31 - line 33 see examples ---	1-7,12, 13
Y	EP,A,0 373 679 (AMGEN INC.) 20 June 1990 cited in the application see claims see page 4, line 23 see page 4, line 35 - line 43 --- -/--	1-13

[X] Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search	Date of mailing of the international search report
1 March 1994	09. 03. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Td. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Scarponi, U

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INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/EP 93/03543

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,Y	EP,A,0 528 314 (BOEHRINGER MANNHEIM GMBH) 24 February 1993 cited in the application see claims 1,3,5,11,13 see page 4, line 26 - line 33 see page 4, line 43 - line 51 see page 5, line 12 - line 17 see page 5, line 40 - line 44	1-13
P,Y	EP,A,0 528 313 (BOEHRINGER MANNHEIM GMBH) 24 February 1993 cited in the application see claims 1,8,10,13 see page 5, line 45 - line 47 see page 5, line 51 - line 53 see page 6, line 40 - line 45 see page 6, line 54 - line 57 see page 8, line 1 - line 2	1-13
P,X	WO,A,93 13752 (SRI INTERNATIONAL) 22 July 1993 see claims 11-14 see page 3, line 19 see page 4, line 9 - line 12 see example 3	1,2,4, 12,13

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. ional Application No
PCT/EP 93/03543

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		JP-A-	63146828	18-06-88
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WO-A-9313752	22-07-93	NONE		

INTERNATIONALER RECHERCHENBERICHT

Internationales Aktenzeichen
PCT/EP 93/03543

A. KLASSIFIZIERUNG DES ANMELDUNGSGEGENSTANDES
IPK 5 A61K37/02 A61K9/14 A61K47/18 A61K47/26

Nach der Internationalen Patentklassifikation (IPK) oder nach der nationalen Klassifikation und der IPK

B. RECHERCHIERTE GEBIETE

Recherchierter Mindestprüfstoff (Klassifikationssystem und Klassifikationssymbole)
IPK 5 A61K

Recherchierte aber nicht zum Mindestprüfstoff gehörende Veröffentlichungen, soweit diese unter die recherchierten Gebiete fallen

Während der internationalen Recherche konsultierte elektronische Datenbank (Name der Datenbank und evtl. verwendete Suchbegriffe)

C. ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie*	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
X	DE,A,37 23 781 (CHUGAI SEIYAKU K.K.) 21. Januar 1988 in der Anmeldung erwähnt siehe Ansprüche 1-5,10-13 siehe Seite 4, Zeile 30 - Zeile 54 siehe Seite 4, Zeile 57 - Zeile 65 siehe Seite 5, Zeile 16 - Zeile 19 siehe Seite 5, Zeile 31 - Zeile 33 siehe Beispiele ---	1-7,12, 13
Y	EP,A,0 373 679 (AMGEN INC.) 20. Juni 1990 in der Anmeldung erwähnt siehe Ansprüche siehe Seite 4, Zeile 23 siehe Seite 4, Zeile 35 - Zeile 43 --- -/--	1-13

[X] Weitere Veröffentlichungen sind der Fortsetzung von Feld C zu entnehmen

[X] Siehe Anhang Patentfamilie

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Datum des Abschlusses der internationalen Recherche	Absendedatum des internationalen Recherchenberichts
1. März 1994	09. 03. 94

Name und Postanschrift der Internationale Recherchenbehörde	Bevollmächtigter Bediensteter
Europäisches Patentamt, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Scarponi, U

Formblatt PCT/ISA/210 (Blatt 2) (Juli 1992)

INTERNATIONALER RECHERCHENBERICHT

Internationales Aktenzeichen
PCT/EP 93/03543

C.(Fortsetzung) ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie*	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
P,Y	EP,A,0 528 314 (BOEHRINGER MANNHEIM GMBH) 24. Februar 1993 in der Anmeldung erwähnt siehe Ansprüche 1,3,5,11,13 siehe Seite 4, Zeile 26 - Zeile 33 siehe Seite 4, Zeile 43 - Zeile 51 siehe Seite 5, Zeile 12 - Zeile 17 siehe Seite 5, Zeile 40 - Zeile 44 ---	1-13
P,Y	EP,A,0 528 313 (BOEHRINGER MANNHEIM GMBH) 24. Februar 1993 in der Anmeldung erwähnt siehe Ansprüche 1,8,10,13 siehe Seite 5, Zeile 45 - Zeile 47 siehe Seite 5, Zeile 51 - Zeile 53 siehe Seite 6, Zeile 40 - Zeile 45 siehe Seite 6, Zeile 54 - Zeile 57 siehe Seite 8, Zeile 1 - Zeile 2 ---	1-13
P,X	WO,A,93 13752 (SRI INTERNATIONAL) 22. Juli 1993 siehe Ansprüche 11-14 siehe Seite 3, Zeile 19 siehe Seite 4, Zeile 9 - Zeile 12 siehe Beispiel 3 -----	1,2,4, 12,13

INTERNATIONALER RECHERCHENBERICHT

Angaben zu Veröffentlichungen, die zur selben Patentfamilie gehören

In...ationales Aktenzeichen
PCT/EP 93/03543

Im Recherchenbericht angeführtes Patentdokument	Datum der Veröffentlichung	Mitglied(er) der Patentfamilie		Datum der Veröffentlichung
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		NL-A-	8701640	16-02-88
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		JP-T-	3502808	27-06-91
		WO-A-	9006762	28-06-90
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		AU-A-	2405292	16-03-93
		WO-A-	9303745	04-03-93
EP-A-0528313	24-02-93	DE-A-	4126983	18-02-93
		AU-A-	2409492	16-03-93
		WO-A-	9303744	04-03-93
WO-A-9313752	22-07-93	KEINE		

E10

RESEARCH ARTICLE

Freeze-Thaw Studies of a Model Protein, Lactate Dehydrogenase, in the Presence of Cryoprotectants

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ABSTRACT: The objective of this study was to investigate the behavior of lactate dehydrogenase (LDH) upon freezing and thawing, alone or in the presence of several selected cryoprotectants. Also, the influence of the freezing rate on retainment of LDH activity was investigated. It was observed that fast freezing caused less loss of LDH activity than slow freezing. The probable mechanisms of loss of activity after freeze-thaw cycles were discussed. Selected cryoprotectants were evaluated for their ability to protect LDH during freeze-thaw cycles. Surface tension and pH change measurements upon freezing of the cryoprotectant solutions were carried out. Based on the results of these experiments, a potential mechanism of cryoprotection has been developed.

Introduction

Many peptides and proteins are being evaluated as potential therapeutic agents. This has been made possible by the significant advancements in biotechnology and related fields. More than two-thirds of all current investigational new drug applications are biotechnology products, and their number is expected to grow from about 2600 in 1991 to about 3250 in 1992 (1). Such changes in the nature of future pharmaceuticals has created a need for the formulation scientist to gain a better understanding of the characteristics and behavior of proteins.

Proteins differ from most conventional drugs in that they have higher molecular weight and a more complex structure. Proteins are composed of some or all of 20 basic amino acids linked together by peptide bonds. The term peptide is used for molecules made of less than 50 amino acid residues while the term protein is used for molecules composed of 50 or more amino acid residues. The complexity of proteins arises because the primary structure (which refers to the specific linear sequence of amino acids) spontaneously folds into secondary and tertiary structures. If the protein has more than one subunit, the interactions between subunits will lead to the formation of a quaternary structure. In a quaternary structure the subunits are held together by non-covalent interactions (2).

Electrostatic, hydrophobic, hydrogen and van der Waal interactions are believed to play a role in protein folding and in creating a functionally active conformation. However, hydrophobic interactions are probably the most instrumental in protein folding (2). Proteins have marginal conformational stability, that is, the difference in the Gibbs free energy of the native and denatured states of protein is about 5–15 KCal/mole (3). This means that even small changes in the environment of the protein may modify the stabilizing interactions leading to the changes in the conformation of the protein. The altered conformation of the protein may have either a diminished or undesirable biological effect or a decreased chemical or physical stability.

Unstable drugs, both conventional and protein, generally can be stabilized to a certain extent by storing the drug at low temperature or by freezing it. However, proteins are not only inactivated at elevated temperatures but may undergo destabilization at low temperatures, termed cold denaturation (4). Adenosine triphosphatase dissolved in sucrose-tris-EDTA solution showed a rapid irreversible decline in activity at 4°C, whereas at room temperature the activity is usually greater. The rate of cold inactivation is normally first order (5). This type of low temperature effect has been seen for several proteins, such as, alcohol dehydrogenase, catalase, chymotrypsinogen, α-glycerophosphate dehydrogenase, immunoglobulin G, myosin, malate dehydrogenase, ovalbumin, phosphofructokinase, phycoerythrin, pyruvate kinase and triose phosphate dehydrogenase (6–9). Low temperature perturbs numerous physico-chemical properties of the proteins and solvents, including pKa of ionizable groups of both the solvent and the protein, and increases the dielectric constant, surface tension, and viscosity of the solvent (10). To gather further insight into the phenomenon of cold denaturation, lactate dehydrogenase (LDH) was selected as a model protein. The primary criterion for choosing LDH was that it is an inherently unstable protein which is inactivated during freezing (11). Also, LDH has been well characterized: its amino acid sequence, putative sites for binding substrate and cofactor, tertiary and quaternary structure and assay procedures have been investigated in detail by several workers.

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LDH (EC 1.1.1.27) is a tetrameric enzyme. There are two types of subunits: M and H, which are coded by two different genes, and they differ in their physical, enzymatic, electrophoretic and immunochemical properties (12). They combine to form five different isozymes. In disease states, the ratio of M to H subunits changes. In muscular dystrophy the concentration of M subunit is lower while in tumors it is higher than the normal level. LDH plays a key role in metabolism (13). The tetramer form of LDH catalyzes the conversion of pyruvate to lactate in the presence of a coenzyme, nicotinamide adenine dinucleotide reduced form (NADH).

The objective of the present study was to investigate the behavior of LDH during freeze-thaw cycle, alone or in the presence of selected cryoprotectants. Further, the influence of rate of freezing on LDH activity was evaluated. It is believed that an understanding of the freezing process and the causes for loss of protein activity during freezing is essential before an optimum freeze-drying cycle can be designed. Based on the results of the following experiments and the concepts recorded in the literature, an attempt has been made to explain the cold denaturation process and how cryoprotectants exert their action.

Materials

L-lactate dehydrogenase, Type II from rabbit muscle, lot 70H9610 was purchased from Sigma Chemical Company (St. Louis, MO). Dipotassium salt of β-nicotinamide adenine dinucleotide, reduced form (lot 40H7180) and pyruvic acid, sodium salt (lot 68F-0752) employed in activity assay were obtained from Sigma Chemical Company. The other chemicals used in the present study were: Methocel E-5 premium (Dow, lot MM84042421E), Tween 80 (Emulsion Engineering Inc., lot 19586-L), protein dye reagent (BioRad, lot 36354), sucrose (J.T. Baker, lot D25331), polyethylene glycol 400 (Spectrum Chemical, lot FF130), Pluronic F-127 (BASF Corporation, lot WPII-529B), dextran (Pharmachem. Corporation, lot 1129-1132), gelatin (BBL, lot H8292-1938-02), D−(+) trehalose (Sigma Chemical Co., lot 110H3834), mannitol (Mallinckrodt, lot 6209 KCLZ), β-cyclodextrin (Roquette, lot E0152), polyvinylpyrrolidone-40 (Sigma Chemical Co., lot 70C-0700), Bovine serum albumin (Sigma Chemical Co., lot 50H9300), Brij 30 (ICI Americas Inc., lot 7762), tris-hydroxymethyl aminomethane (Mallinckrodt, lot 2612 KVKK-2), and sodium chloride (Mallinckrodt, lot 7532 KEEP). All the chemicals were used as such without further purification.

Methods

Characterization and Assay of LDH

Michaelis-Menten constant (Km) of LDH for pyruvate was determined by varying the pyruvate concentration and measuring the velocity of the enzyme catalyzed reaction at 30°C. From the Lineweaver-Burk plot of the data, the Km for pyruvate was calculated to be 0.28 mM. LDH was assayed at 30°C spectrophotometrically on a Perkin-Elmer monochromator (Model lambda 3B). Initial velocities were calculated from the change in the absorbance at 339 nm with time as a result of oxidation of NADH. A typical assay contained 0.23 mM NADH; 81 mM Tris and 203 mM NaCl as Tris-HCl buffer, pH 7.2; and 9.7 mM pyruvate. The reaction was initiated by the addition of 50 μL of solution containing LDH and monitored over 3 minutes period collecting data every 0.5 second using Perkin-Elmer computerized spectroscopy software. LDH activity was calculated by multiplying the initial slope of the line with the factor 9682 to give enzyme activity in U/L (14). See Appendix I for the derivation of the factor.

The protein content of the preparation was determined using the Bradford method of analysis (15). A standard plot was first obtained using bovine serum albumin as a standard. The plot was linear in the concentration range of 0.5 to 25 μg/mL with a correlation coefficient of 0.989.

Influence of Temperature

LDH solution in water was prepared by adding 125 μL of LDH to enough purified water to make 100 mL. The LDH activity and protein content were determined. Samples of 50 mL were kept in screw cap, Type I glass tubes in incubators at 25°C and 40°C and assayed, in triplicate, for LDH activity at selected time intervals.

Rate of Freezing

LDH solution in water was assayed for activity and protein content. One portion was frozen rapidly by dipping 1 mL of the LDH solution in a liquid nitrogen bath (−190°C) for 30 seconds. The other portion of LDH solution was frozen slowly on the shelf of a freeze dryer. This was done by loading the vials at room temperature on the shelf of the freeze dryer, adjusting the shelf temperature to −40°C and then holding for two and a half hours. Both the slow and fast frozen solutions were thawed rapidly by placing the tubes/vials in a water bath set at 30°C, and LDH activity was determined immediately after thawing.

Freeze-thaw of LDH in the Presence of Additives

LDH solution, the protein content having been determined, was mixed with an equal volume of the selected cryoprotectant solution under investigation. All the cryoprotectant solutions used in the study were prepared in water. A mixture of equal volumes of LDH solution and water was used as the control.

Two milliliters of the mixture were pipetted into 10 mL, Type I glass vials and sealed with a butyl rubber closure. For each cryoprotectant under study six such vials were prepared. The LDH activity of each mixture was determined after appropriate dilution, as described previously. Three vials were then placed in the incubator set at 30°C. The remaining three vials were frozen on the shelf of a freeze dryer over a period of 2 hours, bringing the product temperature down to −60°C, after which the shelf temperature was reset to −40°C.

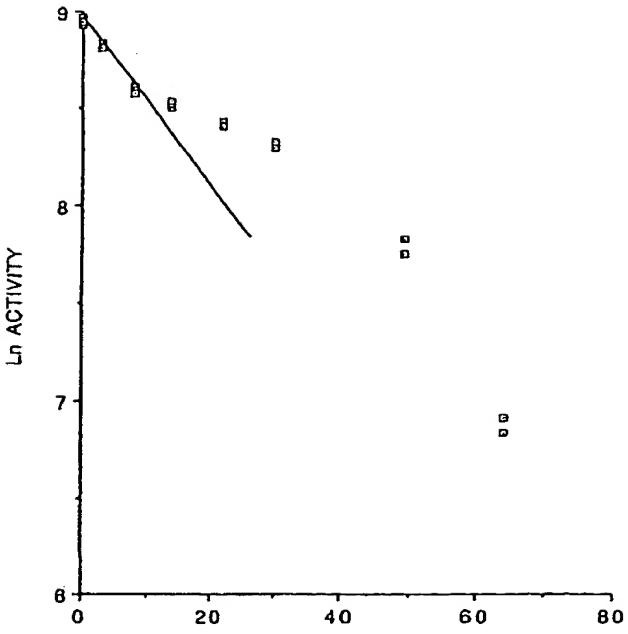

Figure 1—Stability of LDH solution at 25°C. The protein content of the solution was 8.7 μg/mL.

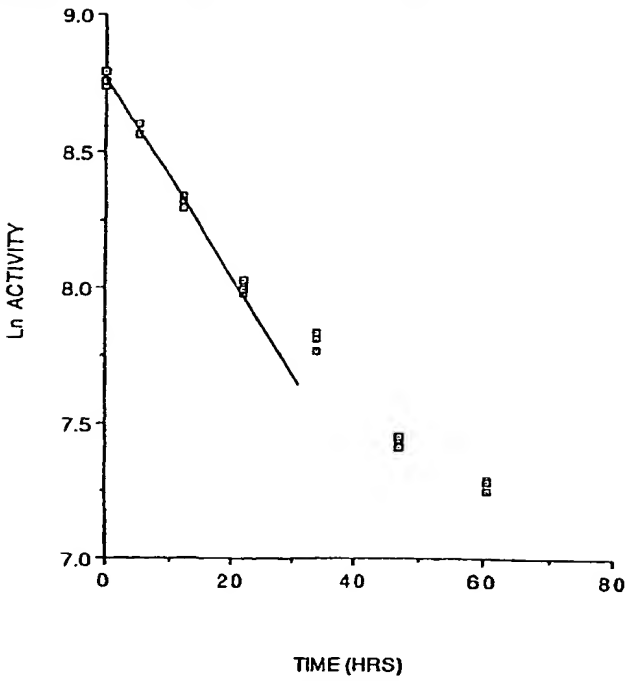

Figure 2—Stability of LDH solution at 40°C. The protein content of the solution was 8.7 μg/mL.

The vials placed in the 30°C incubator were assayed for LDH activity at the end of 21 hours. Vials which were frozen in the freeze dryer were thawed, after being in the freezer for 21 hours, by setting the shelf temperature to 25°C for two hours, followed by placing the vials at room temperature for 1 hour. The thawed samples were immediately assayed for LDH activity.

Changes in the pH of Cryoprotectant Solutions on Freezing

Cryoprotectant solutions were prepared in water at the same concentration as used during the freeze-thaw study. Ten mL of the solution was mixed with 0.5 mL of Universal pH indicator solution. The mixture was placed in 10 mL, Type I, tubing glass vials capped with butyl rubber closures. The color of the mixture was compared to the color on the Fischer universal pH indicator chart. The pH corresponding to the color was noted during the freeze-thaw cycle (16).

The vials were placed on the shelf of the freeze dryer and frozen at −40°C for at least 8 hours. The color of the frozen solution, seen through the transparent plexiglass door, was compared to that on the chart and the corresponding pH recorded. The solutions were then thawed by setting the shelf temperature to 25°C. When completely thawed, the pH of the solution was recorded.

Surface Tension measurements of LDH-cryoprotectant solution

A DuNouy tensiometer (Cenco-DuNouy tensiometer, Model 70545) was calibrated using a 500 mg weight so that each small division corresponded to 1 dyne/cm. Equal volumes of cryoprotectant and LDH solutions were mixed to the same concentrations of LDH and cryoprotectant as employed in the freeze-thaw studies. The force required to break the thin film of solution formed at the platinum ring was measured. The ring was cleaned between successive measurements by dipping it in water, heating it red hot in a Bunsen burner flame and then cooling in air to room temperature.

The surface tension measured by the DuNouy tensiometer is the apparent surface tension. To obtain the true surface tension a correction factor is used. This correction factor includes the circumference of the ring, the density of the liquid under study, and the size of the wire in the ring. The density of the sample solution was determined by pipetting 1 mL of sample into a tared vial and determining its weight using a Mettler balance to give the density in g/mL.

Results and Discussion

Figures 1 and 2 show the plots for the stability of LDH in water at 25°C and 40°C, respectively. The protein

TABLE I
Influence of Rate of Freezing on the LDH Recovery After Freeze-Thaw Cycles

LDH Activity Without Freezing (U/mL)	Activity After Slow Freezing U/mL	Activity After Fast Freezing U/mL
7.4	4.9	6.6
7.5	5.3	6.7
7.8	5.3	6.7

Note: Protein content of the solution was 9 μg/mL.

TABLE II
Recovery of LDH After Freeze-thaw Cycles in the Presence of Different Cryoprotectants

Solutions	Initial Activ.* U/mL	Initial Sp. Activ.** U/mg	21 hrs Activ. U/mL	21 hrs Sp. Activ. U/mg	F/T Activ. U/mL	F/T Sp. Activ. U/mg	% Remain. After 21 hrs	% Remain. After F/T
Water	4.55	429	2.20	208	1.60	151		
	4.50	425	2.32	218	1.66	156	49	36
	4.57	432	2.15	203	1.63	154		
1% w/v Methocel	6.64	626	4.60	434	5.48	514		
	6.69	631	4.51	426	5.57	526	69	82
	6.79	640	4.67	441	5.45	514		
1% w/v Pluronic	5.94	550	3.78	350	5.24	485		
	6.00	556	3.76	348	5.14	467	64	87
	5.95	551	3.87	359	5.23	484		
2.5% w/v PVP	6.02	568	4.67	441	4.71	444		
	6.45	609	5.00	471	4.83	456	77	76
	6.43	607	4.93	465	4.79	452		
0.2m PEG 400	4.46	512	1.59	183	3.81	438		
	4.36	501	1.49	171	3.84	441	35	85
	4.49	516	1.56	180	3.64	418		
0.5% w/v Gelatin	6.03	559	6.45	597	5.42	502		
	6.08	563	6.41	593	5.46	506	106	91
	5.95	551	6.25	578	5.46	506		
1% w/v BSA	5.64	553	5.58	547	6.28	615		
	5.64	553	5.65	554	6.35	623	100	110
	5.76	565	5.80	569	6.08	596		
0.9% w/v	4.71	462	3.24	317	1.46	144		
β-cyclodextrin	4.66	457	3.33	327	1.32	130	71	31
	4.66	457	3.38	332	1.52	149		
5% w/v Dextran	4.81	445	3.32	308	3.60	334		
	4.70	435	3.39	314	3.60	334	71	76
	4.75	440	3.41	316	3.64	337		
5% w/v Trehalose	4.30	422	2.63	258	1.27	124		
	4.46	438	2.63	258	1.06	104	59	26
	4.49	440	2.58	253	1.09	107		
5% w/v Mannitol	4.40	432	3.50	344	0.24	24		
	4.37	428	3.46	339	0.25	24	80	6
	4.36	428	3.56	349	0.28	27		
5% w/v Sucrose	4.19	482	3.24	372	2.97	341		
	4.06	467	3.09	355	3.02	347	77	73
	4.12	473	3.24	373	3.07	353		
10% w/v Sucrose	3.98	457	3.42	393	3.36	387		
	3.84	442	3.41	391	3.57	410	87	88
	3.87	445	3.36	387	3.40	390		
1 M Sucrose	4.08	469	3.07	352	4.26	489		
	4.22	485	3.05	351	4.30	495	73	102
	4.23	487	3.08	354	4.27	491		
0.05% w/v Brij 30	4.63	454	2.12	207	4.66	457		
	4.70	461	2.17	213	4.76	467	46	100
	4.70	458	2.20	216	4.62	453		
2 mg/dL Tween 80	4.75	428	2.86	258	2.71	244		
	4.85	437	2.94	265	2.69	242	61	57
	4.84	436	2.95	266	2.76	249		
5 mg/dL Tween 80	5.11	460	3.10	279	3.37	303		
	5.14	463	3.14	283	3.28	295	60	65
	5.25	473	3.04	274	3.44	310		
0.05% Tween 80	6.00	541	2.20	199	4.71	425		
	6.03	543	2.31	208	4.72	425	37	79
	6.04	544	2.16	195	4.91	443		

Continued on next page

TABLE II (Continued)

Solutions	Initial Activ.* U/mL	Initial Sp. Activ.** U/mg	21 hrs Activ. U/mL	21 hrs Sp. Activ. U/mg	F/T Activ. U/mL	F/T Sp. Activ. U/mg	% Remain. After 21 hrs	% Remain. After F/T
0.1% w/v Tween 80	5.45	491	3.12	281	4.28	386		
	5.55	500	3.04	274	4.42	399	58	81
	5.25	473	3.19	287	4.43	399		
0.25% w/v Tween 80	5.07	456	2.21	200	4.16	375		
	5.03	453	2.05	185	4.26	384	42	84
	5.09	459	2.13	192	4.30	388		
0.5% w/v Tween 80	6.14	579	2.03	192	4.80	453		
	6.02	568	2.08	196	4.90	463	34	80
	6.23	588	2.07	195	4.96	468		
1% w/v Tween 80	5.85	552	2.21	209	4.73	446		
	5.87	554	2.16	204	4.88	460	38	83
	5.61	529	2.20	208	4.80	453		

* Activity has been abbreviated as Activ.
** Sp. Activ. (Specific Activity) is the activity of the enzyme per mg of protein.

content of the solution was 8.7 μg/mL in both cases. These temperatures were selected because 25°C is the normal room temperature and 40°C represents a commonly used elevated temperature. Further, at the temperature of about 50°C a denaturation transition of LDH occurs (as determined by a differential scanning calorimeter). Therefore, the elevated temperature study was carried out at a temperature below 50°C. From the initial points on the degradation curve, first order rate constants were estimated to be 0.045/day at 25°C and 0.034/hour at 40°C. From the rate constants, it can be calculated that 68% of LDH activity will be lost in 11 hours at 40°C while it will take 8 days at 25°C to lose the same amount of activity.

Although the degradation kinetics can be approximated as being first order, there is deviation from linearity when Ln activity is plotted against time. Therefore, the rate constants indicated should not be used as absolute values but as estimates only to project how fast the loss in activity is taking place.

LDH solutions lose considerable activity upon freezing, irrespective of the rate of freezing, as indicated by data in Table I. The molecular basis of cold denaturation may involve changes in the bond strength. At low temperatures, water-protein hydrogen bondings are favored while the hydrophobic bonds become weaker. This may lead to maximum exposure of polar groups of proteins, promoting cold unfolding (17).

When LDH solution was frozen rapidly under liquid nitrogen, more activity was retained compared to freezing slowly on the shelf of the freeze dryer. In both cases, rapid thawing was carried out by dipping the container in a 30°C water bath. It is assumed throughout the discussion that the loss of LDH activity is occuring primarily during freezing and not during the thawing process. The results also point in this direction but there is no definitive evidence to support the conclusion that thawing does not damage LDH activity. The protein content of both the solutions was 9 μg/mL. However, only 68% of LDH activity remained after slow freezing compared to 88% remaining after fast freezing. These results show that even one freeze-thaw cycle results in more damage to LDH activity than storing the solution at 25°C for a week.

There are three mechanisms which have been proposed as responsible for the different activity losses at high and low freezing rates. The first mechanism may involve the shear force exerted on the protein molecules by the ice crystals. It has been reported that smaller ice crystals are formed on rapid freezing and it is possible that they cause less damage to the LDH than larger ice crystals formed during slow freezing (18). Secondly, slow freezing may result in crystallization of water with a concomitant increase in the concentration of protein in the remaining unfrozen solution. This may bring protein molecules into actual physical contact causing aggregation (19). During fast freezing, the solution freezes almost instantaneously, preventing the concentration and ensuing loss of activity. The third phenomenon may involve the weakening of the hydrophobic interactions (6, 17). In aqueous solutions protein tends to minimize unfavorable interactions between its hydrophobic amino acid residues and polar water molecules by folding in such a way that polar residues are generally present at the surface while the non-polar residues are buried in its structure away from the water. If water is trapped inside, it will be in the form of ordered structures. By excluding water molecules from its interior and keeping them to the outside where water is free, the entropy of the system will increase leading toward a thermodynamically stable state. These entropic forces, which are believed to be the most important for the folding of proteins, are called hydrophobic forces. It may be that during slow freezing, the growing ice crystals can extract water from the protein, whereas during fast freezing this is less likely to happen (8, 19). Removal of water from the protein may lead to unfolding or denaturation because the driving force for the protein folding and association of the subunits, the hydrophobic interaction, becomes weaker (6, 17).

LDH, when mixed with selected cryoprotectant solutions, showed varying degrees of protection, either when

held at room temperature or when subjected to freeze-thaw, depending on the nature of the additive. Table II shows the initial activity and specific activity, the activity and specific activity after storing the solution at 25°C for 21 hours, and the activity and specific activity after freeze-thaw cycles. All the samples were prepared and assayed in triplicate. Generally, additives which increased the stability of LDH solution at room temperature also improved the recovery after freeze-thaw. However, this was not always the case, as seen for 0.2M PEG 400 (polyethylene glycol), 0.05% Brij 30 (polyoxyethylene lauryl ether), and all the concentrations of Tween 80 (polyoxyethylene monooleate). Methocel E-5 (hydroxypropyl methylcellulose), Pluronic F-127 (polyoxyethylene-polyoxypropylene copolymer), PVP (polyvinylpyrrolidone), PEG-400, Brij 30, BSA, gelatin, dextran and the higher concentrations of Tween 80 and sucrose were found to be good cryoprotectants during freeze-thaw cycles. The term 'good' is used here to describe those additives, which when added to LDH solution, resulted in about 80% or more recovery of activity after freeze-thaw. A surprising result was obtained with trehalose, a disaccharide (α-D-glucopyranosyl-α-D-glucopyranoside), which is considered by many workers to be one of the best cryoprotectants, but proved to be ineffective in this study at a concentration of 5%w/v.

Different concentrations of Tween 80 were used to evaluate the effect of changes in the cryoprotectant concentrations on the LDH recovery after freeze-thaw cycles. The critical micellar concentration (cmc) for Tween 80 is 3.3 mg/dL. Therefore, the concentration of Tween was varied from below the cmc, just above the cmc, to much higher than cmc concentrations. The cmc concentration did not appear to have a significant relationship to recovery of LDH, however there was more recovery of LDH after freeze-thaw cycles than from the control solution, whether the concentration of Tween 80 was above or below the cmc. The recovery progressively increased as the surfactant concentrations were raised from 2 mg/dL to 50 mg/dL (or 0.05% w/v). Any further increase in the Tween concentration, above 0.05% w/v, did not cause an increase in the recovery or protection. A similar increase in the LDH activity recovery was seen after freeze-thaw cycles as the concentration of sucrose was increased from 5% w/v to 34.2% w/v (1M).

All the additives used in the present study have been reported in the literature to be good cryoprotectants for proteins, especially trehalose, mannitol and sucrose. However, in order to explore why certain additives used in this study protected LDH during freeze-thaw cycles while others did not, changes in the pH of the cryoprotectant solutions with temperature were examined. Since enzymes are susceptible to pH changes, a drift of pH during the freezing step might explain why there is greater loss in LDH activity in some cases (20, 21). Such changes in pH before, upon freezing and after thawing are presented in Table III. The change in pH of cryoprotectant solutions upon freezing was recorded visually, making the procedure subjective. However, in most instances, a pH change of one unit or more (and in many cases as low as 0.5 unit) can be visibly differentiated. The pH of the mannitol solution could not be determined while trehalose and 5% sucrose solutions showed a change in the pH by two units or more upon freezing, compared to the pH before freezing. This might be the reason for the loss in the LDH activity when these cryoprotectants were used. Surprisingly, both trehalose and mannitol provided less protection than the control (water) in protecting LDH activity (see Table II), while 5% sucrose was inferior to higher concentrations of sucrose where there was less change in the pH. Low pH alone did not seem to explain a high loss of activity since 2.5% PVP, 0.5% gelatin, 10% sucrose and 1% w/v Tween 80 solutions had a low pH (≤ 5.5) but provided good protection during freeze-thaw cycling.

TABLE III
Changes in the pH of the Cryoprotectant Solutions on Freezing

Solutions	PH Before Freezing	PH When Frozen	PH After Thawing
Water	7	6.5	7
1% w/v Methocel E5	7	6.5	7
1% w/v Pluronic F127	6.5	6.5–7	6.5–7
2.5% w/v PVP	4	4 to 5	5
0.2 m PEG 400	6.5	6.5	6.5
0.5% w/v Gelatin	5	5	5
1% w/v BSA	7	6.5	7
0.9% w/v B-Cyclodextrin	7.5	6.5	7.5
5% w/v Dextran	7	6	7
5% w/v Trehalose	7.5	5.5	7.5
5% w/v Mannitol	5.5	*	5.5
5% w/v Sucrose	7.5	5.5	7.5
10% w/v Sucrose	7	5.5	7
1 m Sucrose	7	5.5	7
0.05% BRIJ 30	7	6.5–7	7
2 mg/100 ml Tween 80	7.5	6.5	7.5
5 mg/100 ml Tween 80	7	6.5	7
0.05% w/v Tween 80	6.5	6.5	6.5
0.1% w/v Tween 80	6.5	6.5	6.5
0.25% w/v Tween 80	6	6	6
1% w/v Tween 80	5.5	5–5.5	5.5

* pH could not be determined.

It was noticed that some of the good cryoprotectants for LDH were also surface active. Therefore, it was decided to measure the surface tension of each cryoprotectant solution in the presence of LDH. In order to convert the Tensiometer dial reading into surface tension values, a correction factor, which depends on the circumference of the ring, the density of the liquid and the size of the wire in the ring, was employed. When a graph was plotted between percent recovery of LDH after freeze-thaw cycling and surface tension, there was considerable scatter. Therefore, no apparent correlation exists between surface tension and enzyme recovery for these cryoprotectants.

Arakawa, Timasheff and Carpenter have reported that a cryoprotectant should be preferentially excluded from the protein for it to exert a protective effect (22, 23). Exclusion is determined by the effect of addi-

tives on the surface tension of water. Additives perturb the cohesive forces of water and, hence, its surface tension. This results in either an excess or a deficiency of the additive in the protein surface layer. According to this theory, those compounds which increase the surface tension of water should be preferentially excluded from the protein surface and therefore should be good cryoprotectants. In contrast to the work of these authors (24), the present study failed to find any correlation between an additive's effect on surface tension and its protective effect on LDH during freeze-thaw cycles.

Based on the results of the above experiments and a review of the literature, a mechanism of cryoprotection is postulated. The cryoprotectants may change the freezing characteristics of water or modify the nature of ice crystals (25, 26). From the experiments on the rate of freezing, it is possible to conclude that the nature of the ice crystal formed during freezing may influence the recovery of the LDH, since fast freezing, which has been reported to form smaller ice crystals, resulted in higher recovery than slow freezing. Thus, additives which will prevent formation of larger ice crystals or tend to form an amorphous matrix upon freezing, may serve as good cryoprotectants. It has been reported that PEG reduces the ice nucleation temperature and a 37.5% PEG 4000 solution, when subjected to cooling at 1.25°K/min, exhibits no freezing at all (25). Similarly, hydrophilic polymers of high molecular weight, such as PVP, tend to supercool and then solidify without the formation of visible ice crystals (27). Cryoprotectants like PEG, dextran, sucrose, PVP, Pluronic, Methocel and gelatin may modify the freezing behavior of water due to the decreased diffusional freedom of water molecules. The results of the freeze-thaw cycling in this study support this hypothesis. Mannitol, which crystalizes during slow freezing (28), was ineffective in protecting the activity of LDH while PVP, Methocel, PEG and sucrose, which are non-crystallizable, were good cryoprotectants. Also, as the concentration of sucrose was increased, there was an improvement in the protection of LDH activity after freeze-thaw cycles. Higher concentrations of sucrose will probably cause more decrease in the diffusional freedom of water molecules than lower concentrations.

A second mechanism of protection may involve additives like Tween 80, BSA and Brij 30, which have surface active properties and may concentrate at the ice-water interface, preventing the surface denaturation (unfolding and aggregation) of LDH. Also, they may concentrate at the LDH surface, surround the enzyme and protect it from the damaging effects of ice crystals. Polymers like PVP, Pluronic F-127, gelatin, and Methocel E-5, which are surface active and also increase the viscosity of the solution, may exert their protective action by a combination of both mechanisms.

Conclusions

The cold denaturation of protein is an important, but sparingly studied phenomenon. The results from this study show that storing protein solutions under frozen conditions will not always alleviate the stability problem, rather, a single freeze-thaw cycle may cause more activity loss of a protein solution than storing it at room temperature for a week. Pharmaceutical scientists should be aware of this anomalous behavior when performing stability studies on such proteins. Oftentimes the reference solutions are stored in a deep freezer or at least refrigerated, and this practice should be modified for proteins like LDH.

Another implication of cold temperature destabilization is the necessity for using cryoprotectants to prevent the denaturation of protein solutions during freezing or freeze drying. In the present study, selected cryoprotectants were evaluated for their ability to protect LDH activity during freeze-thaw cycles. Several of the cryoprotectants studied did provide good protection for solutions of LDH stored at 30°C and several, not always the same ones, did so when the solutions were subjected to freeze-thaw cycles.

Appendix I

According to Beer-Lambert law:

$A = a\,b\,c$ where A = absorbance,
a = linear millimolar absorption coefficient (L/μmol mm),
b = path length (mm), and
c = concentration (μmol/L)

For any given reaction:

$$\Delta c = \Delta A / a\,b$$

To determine the concentration of enzyme, the ratio of total volume in the cuvette (V) to the volume of enzyme solution (v) added must be taken into account, therefore:

$$\Delta c = \Delta A\, V / a\,b\,v \qquad (1)$$

The catalytic activity of the enzyme (z) is defined as the micromol of substrate converted into product per unit time

$$z = \Delta c / \Delta t \qquad (2)$$

When the unit of time is in minutes and the concentration is in micromol, then the catalytic activity is expressed as U. Substituting equation (1) into (2) we get

$$z = \Delta A\, V / a\,b\,v\,\Delta t \qquad (3)$$

NADH at 339 nm has an absorption coefficient of 0.631×10^{-3} L/μmol mm and the cuvette used in measuring the activity has a path length of 10 mm. The volume of the enzyme solution (v) used for the assay is 0.05 ml or 0.05×10^{-3} L. The total volume (V) added to the cuvette (which includes the volume of NADH, pyruvic acid and enzyme solution) is 3.05 ml or 3.05×10^{-3} L. Inserting these values in equation (3) gives

$$z = (\Delta A/\Delta t)\, 3.05 \times 10^{-3} / 0.631 \times 10^{-3} \times 10 \times 0.05 \times 10^{-3}$$

$$z = (\Delta A/\Delta t)\, 9682$$

($\Delta A/\Delta t$) is determined from the slope of the change in absorbance with time curve.

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